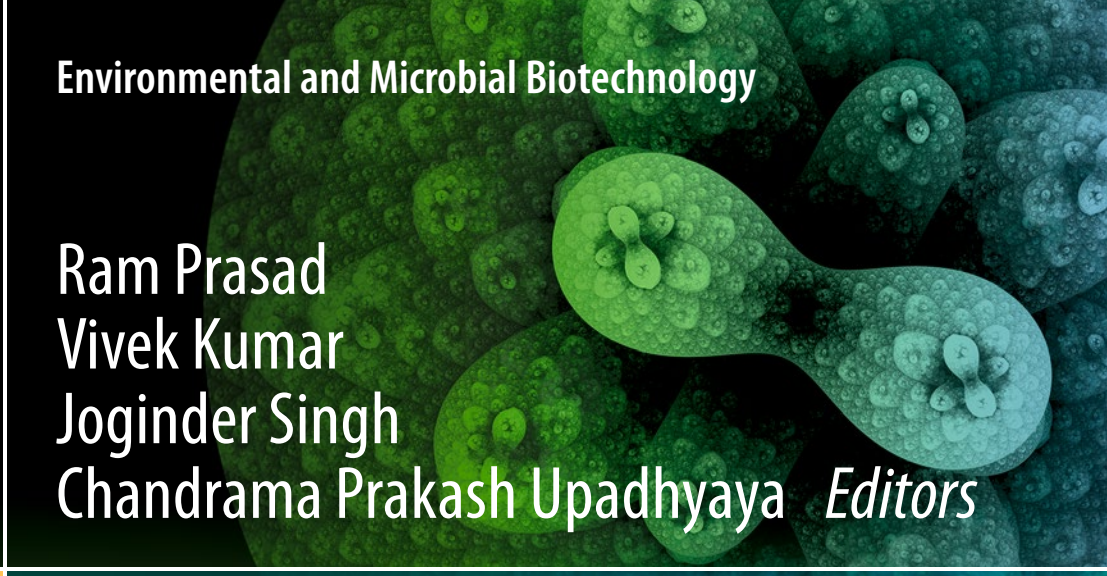
Environmental and Microbial Biotechnology



Ram Prasad
Vivek Kumar
Joginder Singh
Chandrama Prakash Upadhyaya *Editors*

Recent Developments in Microbial Technologies

Springer

Environmental and Microbial Biotechnology

Series Editor

Ram Prasad, Department of Botany, Mahatma Gandhi Central University, Motihari, Bihar, India

Innovative and novel advances in microbial biotechnology are providing great understandings in to the machineries of nature, presenting fascinating prospects to apply principles of biology to different arenas of science. Sustainable elucidations are emerging to address the concerns on improving crop productivity through microbes, depleting natural resources, environmental pollution, microbial degradation of pollutants, nanomaterials, nanotoxicity & safety issues, safety of food & agricultural products etc. Simultaneously, there is an increasing demand for natural bio-products of therapeutic and industrial significance (in the areas of healthcare, environmental remediation, microbial biotechnology). Growing awareness and an increased attention on environmental issues such as climate change, energy use, and loss of non-renewable resources have carried out a superior quality for research that provides potential solutions to these problems. Emerging microbiome approaches potentially can significantly increase agriculture productivity & human healthcare and henceforth can contribute to meet several sustainable development goals.

The main objectives have provided an impetus for research on plants and microorganisms that produce novel bio-products with variable properties and understanding their mechanisms of action at cellular and molecular level. Hence, research activities of the environmental and microbial Biotechnology are comprehensively focused up on major sectors viz., bioresources, biorefining, bioremediation of organic and inorganic pollutants, environmental risk analysis of microorganisms, environmental assessment using microbiological indicators, enzymes for environment, food & industrial applications, nanomaterials & nanotoxicity, sustainable ecobiotechnology, biofertilizer, biocontrol agents for agriculture improvement and natural products for healthcare applications.

This book series is a state-of-the-art for a wide range of scientists, researchers, students, policy makers and academician involve in understanding and implementing the knowledge on environmental and microbial biotechnology to develop biologics for proper health care to continue life in smooth and sustainable strategy without any adverse effect.

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Preface

Over the last few decades, the rapid and immense development of cutting-edge microbial bio-resources and metagenomics techniques has completely transformed the field of microbial biotechnology. With innovative techniques, it is now believed that over 99% of microbes on Earth are uncultured, representing a large untapped biological resource. Our understanding of microbial diversity, evolutionary biology, and microbial interaction with plants, animals, and microbes at the molecular level has been revolutionized with an abundance of new research. The genomics-enabled research on microbes in nature helps us to understand the origin, growth, development, relationships between the biosphere and the environment. Research on microbes thriving in extreme environments has been intensified, yielding a large number of new microbes, some of which are of potential use in various applications.

Our book *Recent Developments in Microbial Technologies* provides insight into the diverse microorganisms that have been explored and exploited in the development of various applications such as plant protection and improvement, environmental remediation, and the improvement of plant and human health. Several applications of microorganisms are covered broadly and have been properly reflected in-depth in different chapters. Microbial technology also includes the production of materials in bioreactors, waste and pollution management, diagnostics and analytical equipment, biosensors, and renewable energy system based on biomass feedstocks. This book also discussed the development of new microbial enzymes and microbes in human health, the detection and exploitation of microorganisms in the diagnosis of human diseases, providing possible holistic approaches to health. This new volume will provide information on new research on the application of microbial biotechnology today.

This volume serves as an excellent reference book for useful to microbial science scholars, especially microbiologists, biotechnologist, researchers, technocrats, and plant biologist. We have honored that the leading scientists who have extensive, in-depth experience and expertise in microbial technology took the time and effort to develop outstanding chapters.

We wish to thank Dr. Naren Aggarwal, Editorial Director; Ms. Aakanksha Tyagi, Senior Editor, Springer; Mr. Ashok Kumar, and Mr. Salmanul Faris Nedum Palli, Project Coordinator, Springer, for generous assistance, constant support, and patience in initializing the volume. Dr. Ram Prasad is particularly very thankful to

Honorable Vice Chancellor Professor Dr. Sanjeev Kumar Sharma, Mahatma Gandhi Central University, Bihar, for constant encouragement. Editors are also very grateful to our esteemed friends and well-wishers and all faculty colleagues of the Mahatma Gandhi Central University, Swami Rama Himalayan University, Lovely Professional University, and Dr. Harisingh Gour Central University, India.

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2020

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Recent Trends in Plant- and Microbe-Based Biopesticide for Sustainable Crop Production and Environmental Security

1

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Abstract

The impact of increasing human population, rising food demand, and adverse effects of climate change, viz., changing rainfall pattern, rising temperature, biotic-abiotic stresses, etc., has tremendously affected global food security. In addition, increased anthropogenic inputs from urbanization, industrialization, as well as outrageous use of chemical fertilizers and pesticides have posed a severe threat to the sustainability of the agroecosystems. For many decades, the use of chemical pesticides against insect and microbial pests has become an integrative part of agriculture and contributed significantly to the crop improvement. But, their long-term persistence, cytotoxicity, and microbial resistance have resulted negative impact on the biosphere, thus creating pollution of diverse ecosystems, land degradation, and biodiversity losses. For the last two decades, alternate pest management strategies have become the new avenues for controlling pest and

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diseases in a greener, safer, and eco-friendly manner. The use of biological control agents (termed as biocides) such as both microbe- and plant-based formulations has been known to be the major emerging tool in crop disease/pest management and appealing alternative to the chemical pesticide in sustainable agriculture. Biopesticides employ the use of naturally occurring substances, i.e., living organisms (natural enemies) or their products (phytochemicals, microbial products) or by-products (semiochemicals) that control pests by nontoxic mechanisms, with high targeted activity against causal agents (insects, fungi, weeds, viruses, nematodes, etc.), and nonpersistence in the environment. The use of biopesticide alone or in combination with agrochemicals has become the new tool in crop protection as a part of biointensive integrated pest management (IPM) strategies. Although biopesticides are slowly substituting the chemical pesticides with great promise, its use to the desired extent is lacking; hence insight on such biological agents is a prerequisite. In this chapter, we have summarized the sources of biopesticides, their plant protective mechanisms (mode of action), availability, and status in India, as well as some critical pros and cons of its use.

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Keywords

Biopesticide · Agroecosystem · Sustainable agriculture · Biocontrol agents · Integrated pest management

1.1 Introduction

In our agricultural system, there are two main challenges in which one is production while second is prevention to which our agriculture is facing day to day. Production refers to the adequate supply of our growing food demands, whereas, prevention deals with environment deterioration by degradation of air, water, and soil which distract and affect our ecosystem (Bashan and Levanony 1990; Brown 1996; Kesavan and Swaminathan 2008; Koul 2011). Diseases, nematodes, insects, rodents, etc. are major concerns of our crops which embody to harm them. Scientists worldwide have considered them as one of the major limitations to stop the extension of our agriculture production (Swaminathan 1989; Zhao et al. 2008; Wang et al. 2009; Godfray et al. 2010; Shiferaw et al. 2011; Thierfelder et al. 2012; Mpandeli and Maponya 2014). These losses were estimated in the major Indian crops depicted in Table 1.1 (Dhaliwal et al. 2010, 2015), while Table 1.2 and Table 1.3 indicated

Table 1.1 Estimated losses to major agricultural crops in India due to insect pest damage

Major agricultural crops of India	[a]Actual production	Approximate estimated loss in yield due to insect pests		[a]Hypothetical production in the absence of losses due to insect pests	[b]Monetary value of estimated losses
		Percentage	[a]Total		
Coarse cereals[c]	19.03	8.00	1.65	20.68	378.20
Cotton	58.17	30.00	24.93	83.10	15767.69
Groundnut	9.71	15.00	1.71	11.43	1172.13
Maize	24.26	18.00	5.33	29.59	1268.41
Other oilseeds[d]	15.16	12.00	2.07	17.23	1215.55
Pulses[e]	19.78	15.00	3.49	23.27	2285.29
Rapeseed-mustard	7.88	20.00	1.97	9.85	1026.70
Rice	106.65	25.00	35.55	142.20	8467.36
Sugarcane	352.14	20.00	88.04	440.18	3160.25
Wheat	93.51	5.00	4.92	98.43	1135.75
Total/ average	706.29	**168.00**	167.95	875.96	**35877.33**

Sources: Dhaliwal et al. (2015)
[a]Million tons
[b]USD million
[c]*Coarse cereals*: jowar, bajra, ragi, barley, small millets
[d]*Other oilseeds*: sunflower, safflower, *Sesamum*, niger seed, soybean, linseed, castor seed
[e]*Pulses*: gram, lentil, arhar, moong, urd

Table 1.2 Economically important and destructive insect pests of major agricultural and horticultural crops

Crop	Common name	Scientific name
Major agricultural crops		
Chickpea	Cutworm	*Agrotis ipsilon* (Hufnagel)
	Pod borer	*Helicoverpa armigera* (Hubner)
Cotton	American bollworm	*H. armigera* Hub
	Pink bollworm	*Pectinophora gossipiella* Saund
	Spotted bollworm	*Earias insulana* Boisd
	Whitefly	*Bemisia tabaci* Genn
Groundnut	Aphids	*Aphis craccivora* Koch
	Groundnut bruchid	*Caryedon serratus*
	Leaf miner	*Aproaerema modicella* Deventer
	Thrips	*Scirtothrips dorsalis* Hood
	Tobacco caterpillar	*Spodoptera litura* (Fab)
Maize	Earworm	*H. armigera* Hubner
	Shoot fly	*Atherigona* spp.
	Stem borer	*Chilo partellus* (Swinhoe)
Pigeon pea	Leaf webber	*Maruca vitrata* (Geyer)
	Pod borer	*H. armigera* (Hubner)
	Pod fly	*Melanagromyza obtusa* (Malloch)
	Pod-sucking bugs	*Clavigralla gibbosa* Spinola
Rapeseed	Aphids	*Lipaphis erysimi* (Kalt)
Rice	Brown plant hopper	*Nilaparvata lugens* Stal
	Gall midge	*Orseolia oryzae* Wood-Mason
	Leaf folder	*Cnaphalocrocis medinalis* Guen
	Stem borer	*Scirpophaga incertulas* Walker
Sesame	Leaf webber	*Antigastra catalaunalis* Dub
Soybean	Girdle beetle	*Obereopsis brevis* (Swed)
	Hairy caterpillar	*Spilosoma obliqua* (Walker)
	Stem fly	*Ophiomyia phaseoli* (Tryon)
Sugarcane	Scale insect	*Melanaspis glomerata* (Green)
	Stem borer	*Chilo sacchariphagus indicus* (Kapur)
Sunflower	Gram pod borer	*H. armigera* Hubner
Tobacco	Tobacco caterpillar	*Spodoptera litura* Fab
	Whiteflies	*Bemisia tabaci* Genn
Wheat	Aphid	*Schizaphis graminum* (Rondani)
Major horticultural crops		
Apple	Codling moth	*Cydia pomonella*
	Phytophagous mites	*Panonychus ulmi* (Koch)
	San Jose scale	*Quadraspidiotus perniciosus* (Comstock)
Grapes	Flea beetle	*Scelodonta stricollis* (Mots.)
	Mealybugs	*Maconellicoccus hirsutus* Green
	Thrips	*Retithrips syriacus* (Mayet)

(continued)

Table 1.2 (continued)

Crop	Common name	Scientific name
Mango	Hopper	*Amritodus atkinsoni* Leth
	Leaf webber	*Orthaga exvinacea*
	Stem borer	*Batocera rufomaculata* Deg
Oranges	Defoliators	*Papilio demoleus*
	Fruit flies	*Carpomyia vesuviana* Costa
Brinjal	Fruit and stem borer	*Leucinodes orbonalis*
Cabbage and cauliflower	Diamondback moth	*Plutella xylostella* Linn
	Tobacco caterpillar	*Spodoptera litura* (Fab)
Tomato	Fruit worm	*H. armigera* Hubner

worldwide destructive enemies of major economically important agricultural and horticultural crops. Therefore, a world-level seminar on "how to reduce the loss of crops in agricultural crops and increase food supply" should be organized by scientists, so that a clear strategy can be prepared to deal with it. The primary systematic study to estimate crop losses by varied pests on a world scale was first done by Cramer (1967). Subsequently, in 2006, an extensive investigation was conducted by Oerke (2006) which aims to calculate the losses in main food and cash crops.

The protection of crops from insects is always done with a purpose which is to protect the crops from the harm done to them, to prevent them to a large extent, or to economically reduce them to an acceptable level. But in the current situation, we are not able to reduce the loss that is suffered from our agricultural crops due to the pests. This is the result of agriculture crops having a lot of losses, and these losses are also increasing rapidly. In such circumstances, we use chemical products to solve this problem; these chemical products include weedicide, pesticides, and fungicide. With the modernization of agriculture, there is also continuous progressive work done by the scientist for the control of these harmful insects of agricultural crops, the protection of crops, and an increase in crop production. In the last few decades, the blind use of pesticides of chemical products is being blown heavily by developed and developing countries, which is increasing and rising every day. The blind use of pesticides of chemical products remains a matter of concern as many problems arise after its use. After the use of these chemical products, whatever residues are left in the field have a very harmful effect on living beings like humans, domestic animals, birds, beelike beneficial insect, etc. In addition, these chemical residues also contaminate our available groundwater (Lacey and Siegel 2000).

Plant- and microorganism-based pesticides and their products have been scientifically proven to be highly effective against the harmful insect pest species (Glare and O'Callaghan 2003; Thakore 2006; Kalra and Khanuja 2007; Gupta and Dikshit 2010; Mazid et al. 2011; Regnault-Roger 2012; Beas-Catena et al. 2014; Olson 2015). The losses of agricultural crops due to insects are being done for a very long time, whose serious impact is lying directly on our agriculture and agricultural practices. The uses of pesticides based on plants and microorganisms are very

Table 1.3 Economically important and destructive diseases of major agricultural and horticultural crops

Crops	Disease name	Pathogen
Major agricultural crop		
Chickpea	Ascochyta blight	*Ascochyta rabiei*
	Botrytis gray mold	*Botrytis cinerea*
	Collar rot	*Sclerotium rolfsii*
	Dry root rot	*Rhizoctonia bataticola*
	Stunt	Bean leaf roll virus
	Wilt	*Fusarium oxysporum* f. sp. *ciceris*
Cotton	*Alternaria* leaf spot	*Alternaria macrospora*
	Anthracnose	*Colletotrichum gossypii*
	Root rot	*Rhizoctonia* spp.
	Verticillium wilt	*Verticillium dahliae*
Groundnut	Aflatoxin	*Aspergillus flavus*
	Crown rot	*Aspergillus niger*
	Early leaf spot	*Cercospora arachidicola*
	Late leaf spot	*Phaeoisariopsis personata*
	Rust	*Puccinia arachidis*
	Stem and pod rots	*Sclerotium rolfsii*
Maize	Charcoal rot	*Macrophomina phaseolina*
	Common rust	*Puccinia sorghi*
	Downy mildew	*Peronosclerospora* spp.
	Fusarium wilt and stalk rot	*Fusarium moniliforme*
	Maydis leaf blight	*Cochliobolus heterostrophus*
Pigeon pea	*Phytophthora* blight	*Phytophthora drechsleri* f. sp. *cajani*
	Sterility mosaic	Sterility mosaic virus transmitted by *Aceria cajani*
	Wilt	*Fusarium udum*
Rapeseed	Alternaria blight	*Alternaria brassicae*
	Downy mildew	*Peronospora parasitica*
	Powdery mildew	*Erysiphe cruciferarum*
Rice	Bacterial leaf blight	*Xanthomonas oryzae*
	Blast	*Pyricularia oryzae*
	Sheath blight	*Rhizoctonia solani*
Sesame	Alternaria leaf spot	*Alternaria sesami*
	Bacterial blight	*Xanthomonas campestris*
	Cercospora leaf spot	*Cercospora sesami*
	Charcoal rot	*Macrophomina phaseolina*
	Phytophthora blight	*Phytophthora parasitica*
	Wilt	*Fusarium oxysporum* f. sp. *sesami*
Soybean	Bacterial blight	*Pseudomonas* spp.
	Bacterial pustule	*Xanthomonas campestris*
	Charcoal rot	*Macrophomina phaseolina*
	Collar rot	*Sclerotium rolfsii*
	Pod blight	*Colletotrichum dematium* f. sp. *truncata*

(continued)

Table 1.3 (continued)

Crops	Disease name	Pathogen
Sugarcane	Red rot	*Colletotrichum falcatum*
	Smut	*Ustilago scitaminea*
	Wilt	*Fusarium sacchari*
Sunflower	*Alternaria* blight	*Alternaria helianthi*
	Gray mold	*Botrytis cinerea*
	Scorch	*Macrophomina phaseolina*
	Wilt	*Verticillium dahliae*
Tobacco	Damping-off	*Pythium aphanidermatum*
	Frogeye spot	*Cercospora nicotianae*
Wheat	Karnal bunt	*Neovossia indica*
	Leaf or brown rust	*Puccinia recondita* f. sp. *tritici*
	Loose smut	*Ustilago segetum*
	Stem or black rust	*Puccinia graminis* f. sp. *tritici*
Major horticultural crops		
Apple	Scab	*Venturia inaequalis*
Grapes	Anthracnose	*Gloeosporium ampelophagum*
	Downy mildew	*Plasmopara viticola*
	Powdery mildew	*Uncinula necator*
Mango	Anthracnose	*Colletotrichum gloeosporioides*
	Powdery mildew	*Oidium mangiferae*
Oranges	Canker	*Xanthomonas campestris* pr. *citri*
	Gummosis	*Diaporthe citri*
Brinjal	Damping-off	*Phytophthora* spp. or *Pythium* spp.
	Wilt	*Fusarium ozonium*
	Phomopsis blight	*Phomopsis vexans*
Cabbage	*Alternaria* blight	*Alternaria solani*
	Black rot	*Xanthomonas campestris*
	Downy mildew	*Peronospora parasitica*
Cauliflower	Stalk rot	*Sclerotinia sclerotiorum*
Tomato	Late blight	*Phytophthora infestans*
	Leaf blight	*Septoria lycopersici*
	Tomato spotted wilt	Vial disease
	Wilt	*Pseudomonas solanacearum*

effective against these harmful factors of agriculture such as pests, fungus, weed, viruses, nematodes, animals, and birds. Besides, they are very environment-friendly in nature. In this way, pesticides based on plants and microorganisms are leading around the world as strategies for adoption of insect and pest management (Koul 2011).

The plants and microorganisms have a long history of study and are considered to have a great potential candidate in insect and pest management. The biopesticides are bound forms of pesticides derived from such natural materials as animals, plants, bacteria, and bound minerals. As an example, canola oil and baking soda have

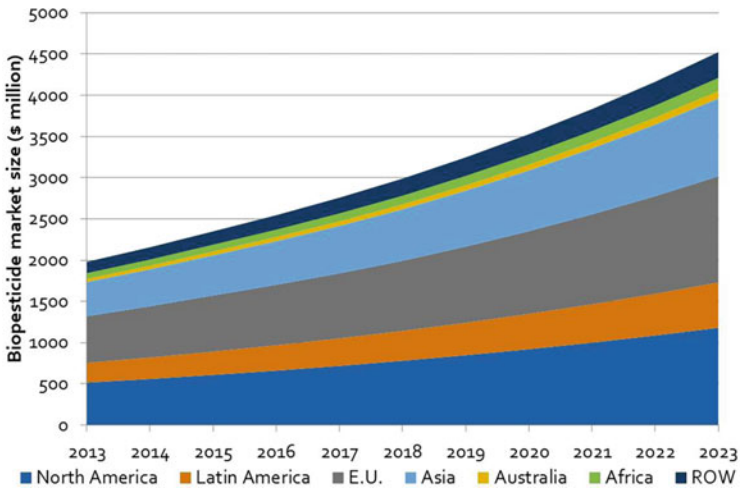

Fig. 1.1 Global market of biopesticides from 2013 to 2023 (Olson 2015)

pesticidal applications and are considered biopesticides. Even at the end of 2001, there were approximately 195 registered biopesticide active ingredients and 780 products (OBPPD 2018). A report entitled "An Analysis of the Biopesticide Market Now and Where It Is Going" was published by a research analyst in the US-based company "Lux Research Inc.," in which it was stated that the global market of biopesticides was approximately $3 billion with a small fraction of the total world's crop protection in 2013 and estimated at $4.5 billion by 2023 (Olson 2015) (Fig.1.1). Biopesticides are organic chemistry-based pesticides that are present naturally as substances that do the management of pests by their nontoxic mechanisms. They are living organisms (natural enemies) or their product (phytochemicals, microbial products) or by-products (semiochemicals) which might be used for the control of pests that are injurious to crop plants (Table 1.4). Biopesticides have a vital role in crop protection, though most ordinarily together with alternative tools as well as chemical pesticides as a part of biointensive integrated pest management (IPM) (Magid et al. 2011; Usta 2013).

1.2 Biopesticide at a Glance

Biopesticides are derived from algae, bacteria, fungi, nematodes, protozoa, and virus, and some other compounds made directly from these microbes like metabolites are main microbial control agents of insect pest (Van Lenteren 2012), for instance, a combination of canola oil and baking soda which has pesticide applications. They are used in agriculture to control various pests, pathogens, and weeds. *Trichoderma* as biofungicides, *Phytophthora as* bioherbicides, and *Bacillus thuringiensis* (*Bt*) as bioinsecticides come to be known as biopesticides (Gupta and Dikshit 2010). Biopesticides or biological pesticides provide an ecologically very

Table 1.4 Potential microbe-based biopesticides with their target organisms

Potential biopesticides	Taxonomy	Targets	Commercial names
Ampelomyces quisqualis	Fungus	Powdery mildew	*Bio-Dewcon*
Bacillus thuringiensis subsp. *kurstaki*	Bacterium	Lepidopteran pests	*Bio-Dart*, *Halt*, *Biolep*
Bactericides; *B. subtilis*	Bacterium	Soilborne pathogens	–
Beauveria bassiana	Fungus	Coffeeberry borer, grasshoppers, aphids, codling moth	*Myco-Jaal*, *bio-larvex*, *bio-grubex*, *ATEC Beauveria*
H. armigera (NPV)	Virus	*H. armigera*	*Helicide*, *bio-virus H*, *HeliGuard*
Insecticides; *B. thuringiensis* subsp. *israelensis*	Bacterium	Lepidopteran pests	*Tacibio*, *Technar*
Nematicides; *Verticillium chlamydosporium*	Fungus	Nematodes	–
Pseudomonas fluorescens	Bacterium	Plant soilborne diseases	Biomonas, Sudo, Sun Agro Monus
Spodoptera litura	Virus	*Spodoptera litura*	*Spodocide*, *bio-virus S*
Trichoderma harzianum	Fungus	Soilborne pathogens	*Biozim*, *Sun Agro*, *Derma H*
T. viride	Fungus	Soilborne pathogens	*TrichoGuard*, *ectoderm*, *defense*

effective solution to the problems of pests in which they are very specific only for their target insect pest.

These are eco-friendly pesticides which are obtained from naturally occurring substances (biochemical), microbes, and plants. Biopesticides have come into the light due to several disadvantages related to the application of chemical pesticides like genetic variations in plant populations, reduction of useful species, environmental pollution or water contamination, food poisoning, and health-related issues like cancer. The total world production of biopesticides is over 3000 tons/year. India has a vast potential for biopesticides. *Bt*, NPV, neem-based pesticides, *Trichoderma*, etc. have been already registered in India (Kandpal 2014).

Agriculture plays a vital role in a developing country like India. It also plays a role in improving the economy of the country. So, the use of biopesticides can play a very important role to deal with all challenges in a sustainable manner (Gupta and Dikshit 2010). There are some available examples of biopesticides and biocontrol agents which have been successfully utilized in Indian agriculture (Shia and Feng 2004). Among them, some are as follows: diamondback moth control by *Bt*, rot and wilt control in several crop by-products based on *Trichoderma* formulation, sugarcane borer control by *Trichogramma*, whitefly control in cotton by neem products, etc. (Weibin and Mingguang 2004).

1.3 Classification of Biopesticides on the Basis of Plant and Microbe Origin

1.3.1 Biopesticides of Plant Origin

1.3.1.1 Plant Pesticides

To decrease crop destruction by phytophagous arthropod pests, one strategy is to genetically modify (GM) plants to express specific genes that encode insecticidal toxins. In the last 11 years, the acceptance of GM crops has risen dramatically. GM crops have a gene or genes transmitted from a distinct species. In 1996, the production of transgenic crops expressing insecticidal δ-endotoxins obtained from the soil bacterium *Bt* was first marketed in the United States of America (USA) (Sanahuja et al. 2011). These toxins' expression provides auspice against insect crop destruction. The lethality of *Bt* endotoxins is extremely dependent on the insect intestine's alkaline environment, which ensures that these toxins are not active invertebrates, particularly in humans (Zhang et al. 2006). These proteins were generated commercially, targeting significant cotton, maize, potato, rice, tobacco, and tomato pests, particularly enabling higher coverage by achieving places on crops inaccessible to foliar sprays. There are various *Bt* strains each with distinct Cry proteins, and they have recognized more than 60 Cry proteins. Most *Bt* maize hybrids express the Cry1Ab protein, and some express the Cry1Ac or the Cry9C protein, all targeting the European maize borer (*Ostrinia nubilalis* Hubner) (Lepidoptera), the main maize pest in North America and Europe. Some recent hybrids of maize express Cry3Bb1 protein aimed to target the corn rootworm complex [*Diabrotica* spp. Coleoptera], also known particularly for North America as a main pest of maize, while the protein Cry1Ac is expressed by cotton which is aimed against the insect cotton bollworm [*Helicoverpa zea*, Lepidoptera] (Gomez et al. 2002).

1.3.1.2 Botanical Biopesticides

The first botanical insecticide used to kill plum beetles was nicotine, derived from the leaves of tobacco in the seventeenth century. Undoubtedly, the plant kingdom is a storage center for diverse secondary metabolites (e.g., coumarins, flavonoids, phenols, quinones, saponins, sterols, and terpenoids), which are synthesized by the crops themselves and accommodated as defensive weapons against pest attack. These secondary metabolites show varying efficacy against pest species (Raja 2014).

The use of botanicals has become an important means for the protection of crops and the environment and also prevention of pollution of chemical pesticide, which is known as an international problem. These are obtained from either entire plants or their different parts which have the capacity to kill insects, sterilize, prevent weeds, and regulate plant growth. More than 6000 species of plants with insecticidal characteristics have been notified. A number of plant products are obtained from various plants (e.g., custard apple, neem, pyrethrum, tobacco, etc.) which are used as safer insecticides for insect pest management (Koul 2012) because all of them contain some active chemical compounds which have a mechanism to kill the insect

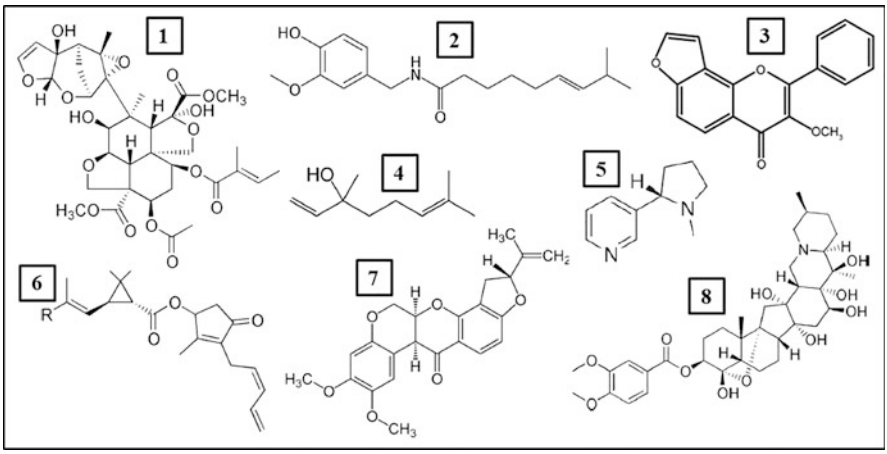

Fig. 1.2 Chemical structure of active compounds of botanical pesticides. 1) Azadirachtin A, 2) capsaicin, 3) karanjin, 4) linalool, 5) nicotine, 6) pyrethrin [If I then R = CH_3, and If II then R = CO_2CH_3], 7) rotenone, and 8) veratrine

Table 1.5 Toxicity level of active chemical compounds of some important botanical pesticides (in mg/kg)

Generic name	Oral LD_{50}	Dermal LD_{50}
d-Limonene	> 4.000	> 5.000
Linalool	2.440–3.180	3.578–8.374
Neem oil	> 5.000	> 2.000
Nicotine	50–60	50
Pongam oil	> 4.000	> 2.000
Pyrethrins	1.200–1.500	> 1.800
Rotenone	60–1.500[a]	940–3.000
Ryania	750–1.200	4.000
Sabadilla	4.000	–

Sources: Pavel (2016)

pest. The structures of these active chemical compounds are given in Fig. 1.2. These compounds of botanical pesticides have wide variation in their toxicity level and are presented in Table 1.5. Azadirachtin-derived compounds from the neem tree are marketed under different trade names and can be used to control scale, thrips, whitefly, and other pests, in many agricultural crops and ornamental plants (Sarwar et al. 2012; Sarwar et al. 2013).

The conventional insecticide has intrinsic toxicity that puts farm operators and the environment at risk for their health. Because of their minimal expenses and ecological side effects, these adverse impacts on human health resulted in a renewed interest in botanical insecticides (Khater 2012). Due to their biodegradation in nature, various mode of action on target pests, and not leaving of toxic residues, botanical pesticides are regarded as one of the eco-safe options. These are potential future

sources for the growth of environment-friendly crop protection goods (Raja 2014). Some of them are commercially available botanical pesticides which are produced and used worldwide for sustainable crop production, Table 1.6.

1.3.2 Microbe-Based Biopesticides

Man developed an interest in victimization of microorganism primarily based on biopesticides once studies on epizootics (entomopathogenic bacteria and fungi) were done throughout the latter half of the nineteenth century and the half of the twentieth century. These microbe-based biopesticides contain a microorganism; it may be bacterium, fungus, virus, protozoan, or algae as the active ingredient (Pandey et al. 2010). Ravensberg (2011b) recommended significant steps for the screening process of suitable isolate microbial strain for the development of commercial production of microbe-based biopesticide (Fig. 1.3)**.** They control different kinds of pest – specific to their target. Table 1.7 indicates some of the examples of microbe-based biopesticides.

1.3.2.1 Bacterial Biopesticides

Bacterial biopesticides are the furthermost extensively known microbe-based biopesticides. There are ranges of *Bt* bacterium which have been identified as insect pathogens and have got maximum importance as a microbial agent over hundreds of bacteria. This bacterium produces a specific protein and is recognized as very harmful to control certain insect pests in cabbage, potatoes, and other agricultural crops (Nawaz et al. 2016). The spores of *Bt* bacterium were the primary and widely used biopesticide within the history of bacterial biopesticides. The spore of *Bt* was isolated from a pathological silkworm (*Bombyx mori*) in 1901 by Japanese scientist Shigetane Ishiwata (Ishiwata 1901). It was found 10 years later by Ernst Berliner in Thuringia, Germany, in a diseased flour moth caterpillar (*Anagasta kuehniella*) (Berliner 1915). In 1911, the *Bt* pathogen was categorized as *B. thuringiensis*-type species (Crickmore et al. 1998). And *Bt* pathogen continues to be the most commonly used biopesticide to date. The French started to apply *Bt* as a biological insecticide in the early 1920s. Sporeine, the first commercial *Bt* product, was introduced in France in 1938. In the year 1977, *B. thuringiensis* var. *israelensis* (fly toxic) was found, and tenebrion strain (beetle toxic) was found in 1983. Finally, in the 1980s and 1990s, the products were developed on commercial scale which included other bacteria such as *P. fluorescens* and *Agrobacterium radiobacter* for the prevention of fire blight and crown gall in orchards and woody crops, respectively. Since the mid-twentieth millennium, bacteria have been used in biological management of insect pest, but very few entomopathogenic bacteria have been established as commercially accessible biopesticides. Presently, *Bt* is one of more than 90% of all biopesticides used and marketed globally. About more than 100 products based on *Bt* have been developed and jointly used against at least 1000 species of insect pest (Glare and O'Callaghan 2003). The *Bt* species consists of various types and subspecies, which can generate a broad range of invertebrate-specific toxins (Lecadet et al. 1999; Glare and O'Callaghan 2000).

Table 1.6 Most commonly worldwide-produced plant-based (botanical) pesticides on a commercial scale

Species	Family	Active compounds	Example of commercial products	Company name	Mode of action
Allium sativum L.	Liliaceae	Sulfur compounds, e.g., diallyl trisulfide, diallyl disulfide, methyl allyl trisulfide	AjoNey	Invernaderos Hidroponicos Neisi, Mexico	The action mechanism of garlic is nontoxic for the repulsion of insects and birds
			EcoA-Z®, L'EcoMix®, or CapsiAlil®	Ecoflora Agro, Colombia	
Annona squamosa L.	Annonaceae	Squamocin (annonin), debitterized annona oil	ANOSOM®	Agri Life, India	Studies on the mechanism of action have shown that dunnione has insecticide and fungicidal activity by means of mitochondrial complex III inhibition
Azadirachta indica Juss.	Meliaceae	Azadirachtin, salannin, nimbin	MARGOSOM®	Agri Life, India	Azadirachtin (and other tetraterpenoids) inhibits AChE activity in insects considerably. AChE-EC is the main enzyme to stop the nervous impulse by catalyzing nervous system neurotransmitter hydrolysis. Azadirachtin has recently been classified as an antimitotic (G2/M cell division stage) insecticide. Radiolabeling azadirachtin studies have shown their particular corpus cardiac locations
			Molt-X®	BioWorks, Inc., USA	
			NeemAzal T/S	Trifolio-M, Germany	
			AZERATM	MGK®, USA	
Capsicum annuum L.	Solanaceae	Protoalkaloids, e.g., capsaicin	Hot Pepper Wax	Rincon-Vitova Insectaries, USA	Capsaicin in insects causes a metabolic disturbance, damage to the membrane, and failure of the nervous system. It also has a repellent physical intervention
			ChileNey	Invernaderos Hidroponicos Neisi, Mexico	

(continued)

Table 1.6 (continued)

Species	Family	Active compounds	Example of commercial products	Company name	Mode of action
Celastrus angulatus Maxim.	Celastraceae	Sesquiterpene pyridine alkaloids	CELAN-X SL	Marketing Arm International, Inc., USA	Not identified
Citrus × *sinensis* (L.) Osbeck	Rutaceae	Limonene and linalool	Demize EC	Paragon Professional Pest Control Products, USA	The neurotoxic mode of action, most prominent symptoms are hyperactivity followed by hyperexcitation that leads to fast knockdown and immobilization. For linalool, acetylcholinesterase inhibitor was recognized
			PREV-AM	Oro Agri SA (Pty) Ltd., South Africa	
Chrysanthemum cinerariaefolium (Trevir.) Vis.	Asteraceae	Pyrethrins (cinerins, jamolins, and pyrethrins)	Spruzit®	Neudorff, Germany	Pyrethrins exert their poisonous impacts by disrupting the process of exchanging sodium and potassium ions in nerve fibers of insects and interrupting ordinary nerve impulse transmission. Insecticides with pyrethrins act incredibly quickly and cause instant paralysis in insects
			PyGanic® Crop Protection EC 5.0 or AZERATM	MGK®, USA	
			1.5% Aphkiller AS	Beijing Kingbo Biotech Co., Ltd., China	
Lonchocarpus spp., *Derris* spp.	Fabaceae	Rotenone	5% rotenone ME	Beijing Kingbo Biotech Co., Ltd, China	Rotenone acts as a cellular respiration inhibitor (a mitochondrial complicated electron transportation inhibitor), causing the feeding to cease rapidly. After exposure, death takes place several hours to a few days
			Rotenone dust	Bonide Products, Inc., USA	

Nicotiana tabacum L.	Solanaceae	Nicotine	Nico Dust or Nico Neem	Nico Orgo Manures, India	It competes with acetylcholine, the main neurotransmitter, by binding to nerve synapses with acetylcholine receptors and causing uncontrolled firing of the nerve. This disruption of ordinary nerve impulse activity results in a fast failure of those body systems that rely on nervous input to function properly
			10% nicotine AS	Beijing Kingbo Biotech Co., Ltd., China	
Pongamia pinnata (L.) Pierre	Fabaceae	Karanjin, debitterized karanjin oil	DERISOM®	Agri Life, India	Pongam oil is a complicated mixture of biologically effective components, and the precise methods of operation of different components or procedures are hard to identify. It is most effective in insects as a killing deterrent but also acts as a repellent, development controller, oviposition (egg formation) suppressant, or sterilizer in multiple types
			Rock Effect	Agro CS a.s., Czech Republic	
Schoenocaulon officinale A.Gray	Melanthiaceae	Cevadine; veratridine	Veratran D®	MGK®, USA	In insects, toxic alkaloids of sabadilla influence the action of the nerve cell membrane, resulting in failure of nerve cell membrane activity, reduction of nerve function, paralysis, and mortality

Sources: Pavel (2016)

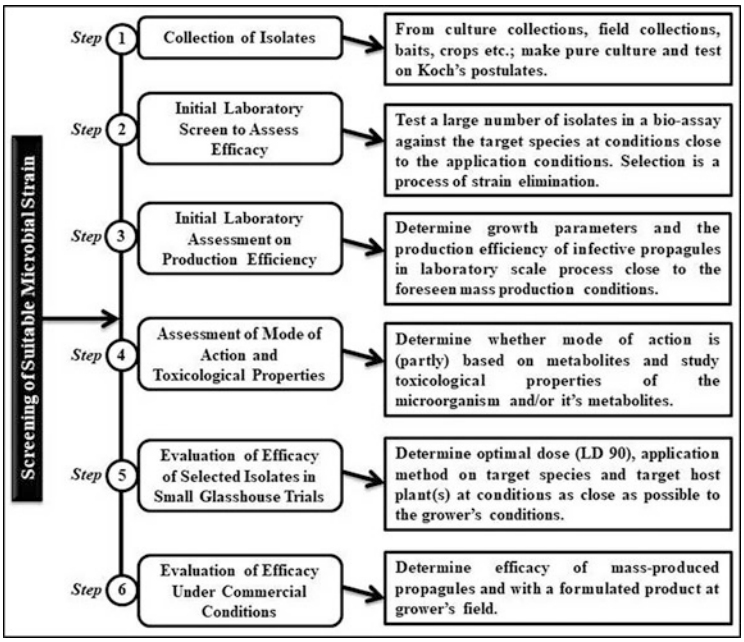

Fig. 1.3 Recommended steps toward screening of an isolate for development of biopesticide to control insect pest for sustainable crop production (Ravensberg 2011b)

Table 1.7 Microbe-based pesticides with the mode of action and their examples

Biopesticides	Mode of action	Examples
Bacteria	Toxins are produced that are harmful if ingested by certain pests	*Bt* (*Bacillus popilliae*), *Agrobacterium radiobacter*
Fungi	Insect controls through growth and secretion of enzymes that weaken the outer coat of the insects]	*Entomophaga praxibulli*, *Zoophthora radicans*, *Neozygites floridana*
Nematodes	By entering natural body openings or directly penetrating the insect cuticle, they destroy their target organisms	*Heterorhabditis bacteriophora*, *Phasmarhabditis hermaphrodita*
Protozoa	When ingested, this kills insects. Food behavior of insects is interrupted, so it starves and dies	*Vairimorpha*, *Malamoeba*, *Nosema*
Viruses	Insects are killed when they are ingested; insect feeding is disrupted, so it starves and dies	Entomopox

B. thuringiensis is a soil-dwelling gram-positive bacterium widely used as a biopesticide. *B. thuringiensis* is also naturally present in the gut of caterpillars of several types of butterflies and moths. Many strains of *Bt* produce crystal proteins (proteinaceous inclusions) during sporulation called δ-endotoxins, which act as

insecticides. When a bacterium is ingested by an insect, alkaline conditions and activity of an enzyme in the intestines activate the protein toxin. The activated toxin then binds to receptor sites and destroys the intestinal wall cells of the insect, allowing the intestinal content to enter the body cavity and bloodstream of the insect. Poisoned insects may die quickly as a result of toxin activity or may die within 2 or 3 days. A few days before the insect dies, it stops feeding and thus stops damaging plants shortly after the ingestion of *Bt*. *Bt* does not colonize in the environment because they get inactivated in the soil with a pH below 5.1. Figure 1.4 describes the mechanism of their toxin's action against the insects of the Lepidoptera order. *Bt* is categorized as immobile because they do not have the ability to leach or move with the groundwater and do not adversely impact the aquatic systems. These products' square measure is accessible as wettable powders, liquid concentrates, and ready-to-use granules and dust. They have been applied for the management of numerous common leaf-feeding caterpillars, e.g., worms that attack broccoli, Brussels sprouts, cabbage, cauliflower, etc. (Table 1.8).

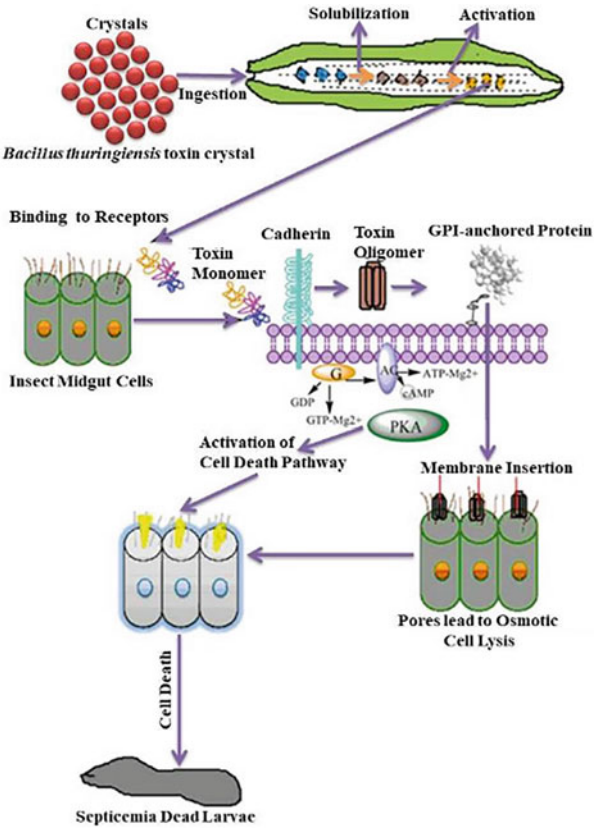

Fig. 1.4 Action mechanism of *Bt t*oxins against insects of Lepidoptera order (Senthil-Nathan 2015)

Table 1.8 Development of different transgenic crops against insect resistance based on *B. thuringiensis* to avoid crop losses

Crops	Gene	Target insect pest	Effects of *Bt* application	Reference
Alfalfa	Cry 1C	Leafworm	–	Strizhov et al. (1996)
Brassica	Cry 1 A(c)	Pod borer	–	Stewart Jr. (1996)
Corn	Cry 1 A (b)	European corn borer	–	Koziel et al. (1993); Armstrong et al. (1995)
		Corn earworm (*H. zea*)	–	Sims et al. (1996)
		2^3 Lepidoptera	–	Pilcher et al. (2005)
	Cry 1 A (c)	Corn earworm (*H. zea*)	Decrease of feeding and survival	Walker et al. (2000)
		Lesser cornstalk borer (*Elasmopalpus lignosellus*)	Decrease of feeding and survival	Walker et al. (2000)
Cotton	Cry 1 A (b)/(c)	Lepidoptera	–	Stewart et al. (2001); Chiticowski et al. (2003)
	Cry1A	Pink bollworm	–	Wilson et al. (1992)
	Cry2A		–	Tabashnik et al. (2002a, 2002b)
Potato	Cry 5	*B. thuringiensis* potato tuber moth	–	Douches et al. (1998)
	Cry1A	Tuber moth	–	Peferoen (1992)
	Cry3A	Colorado potato beetle	–	Perlak et al. (1993)
Soybean	Cry 1 A (b) native	Soybean caterpillar (*Anticarsia gemmatalis*)	Prevention of larvae feeding and growth	Parrott et al. (1994)
	Cry 1 A (c)	Bollworm and budworm	–	Stewart Jr. (1996)
		Soybean caterpillar (*Anticarsia gemmatalis*)	100% mortality to *A. gemmatalis*	Stewart Jr. (1996)
		Soybean looper (*Pseudoplusia includens*)	Decrease of feeding and survival	Walker et al. (2000)
Sugarcane	Cry 1 A (b)	Stem borer	–	Arencibia et al. (1997)

(continued)

Table 1.8 (continued)

Crops	Gene	Target insect pest	Effects of *Bt* application	Reference
Tobacco	Cry 2aa2	Cotton bollworm	–	De Cosa et al. (2001)
	Cry1A	Heliothines	–	Warren et al. (1992)
		H. zea	–	Hoffmann and Frodsham (1993)
Tomato	*B. thuringiensis* (k)	Tobacco hornworm, tomato pink worm, and tomato fruit worm	–	Delannay et al. (1989)

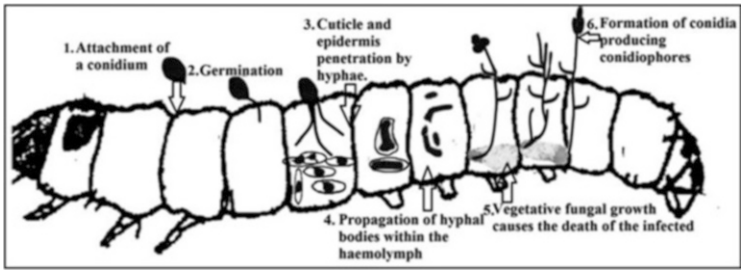

Fig. 1.5 Action mechanism of entomopathogenic fungus against insects of Lepidoptera order (Senthil-Nathan 2015)

1.3.2.2 Entomopathogenic Fungi as Biopesticide

The term "entomogenous" was derived from two Greek words "entomon" meaning insects and "genes" meaning that arose in. Entomogenous microorganisms are, therefore, "microorganisms that occur in insects." Due to their broad host range, entomopathogenic pillows are possibly the most versatile biological control agents. These pillows are naturally occurring organisms that are considered to be less environmentally harmful (Rai et al. 2014). An entomopathogenic fungus is as a fungus that can behave as an insect parasite and kill or severely disable it. Figure 1.5 describes the mechanism of entomopathogenic fungus's action against the insects of the Lepidoptera order. Entomopathogenic fungi trigger deadly diseases, and epizootics control the population of insects and mites in nature (Burger 1981; Carruthers and Soper 1987; McCoy et al. 1988). They infected a broad variety of insects, which included lepidopterous larvae, aphids, and thrips (Roberts and Humber 1981).

"Boverin" (marketable mycoinsecticide) based on *B. bassiana* with reduced doses of trichlorphon is used to suppress *Cydia pomonella*. The fungus *Metarhizium anisopliae* is effective against adult *Aedes aegypti* and *Aedes albopictus* mosquitoes (Shahid et al. 2012). These fungi are widespread and prevalent in nearly all insect

groups. Approximately 750 species of fungi are being developed as pathogens against insect pests. Usually, these are recognized on the basis of development of insect corpses. Entomopathogenic fungi were intended to develop in almost all groups of insects as inundative biocontrol agents of insects, mites, and ticks (Butt et al. 2001; Goettel et al. 2005). These fungi's difference in pathogenicity from bacteria and viruses is that they infect insects by breaching the host cuticle and by secreting extracellular enzymes like chitinases, lipases, and proteases to degrade the major constituents of the cuticle (i.e., lipids, chitin, and protein) and permit hyphal penetration (Wang et al. 2005; Cho et al. 2006). Many toxic substances such as small secondary metabolites, cyclic peptides, and macromolecular proteins are recorded from entomopathogenic fungi. *B. bassiana* produces small molecular weight cyclic peptides and cyclosporines A and C with insecticidal characteristics such as brassinolide, beauvericin, and enniatins (Roberts 1981; Vey et al. 2001).

1.3.2.3 Viral Biopesticides

Viral biopesticides are a kind of pathogens that attack and kill insects and alternative arthropods. They are an alternative like other microbes which are nowadays frequently utilized in biopesticide development for sustainable agriculture. These do not seem to be classified as a living organism but rather as parasitically replicating microscopic particles. Baculovirus is one of the examples which consists of ds-DNA. Due to this genetic material, it can be simply dismantled if exposed under sunlight or other unfavorable conditions within the host's gut. *Heliothis* NPV was approved as the first viral insecticide. The nuclear polyhedrosis virus (NPV) that refers to the baculovirus class is a virus that affects insects, mainly moths, and butterflies. Some important NPVs are listed in Table 1.9. It has been used as a biopesticide by Lasa et al. (2007) for crops in southern Spain and reported having more efficiency than the chemical insecticide (Fig. 1.6). This virus is very species-specific and is a polyhedral crystal that protects the virus outside of the environment

Table 1.9 List of nuclear polyhedrosis virus (NPV)

Nuclear polyhedrosis virus (NPV)	Abbreviations	Nuclear polyhedrosis virus (NPV)	Abbreviations
Autographa californica MNPV (type species)	AcMNPV	*Orgyia pseudotsugata* MNPV	OpMNPV
Anticarsia gemmatalis MNPV	AgMNPV	*Orgyia pseudotsugata* SNPV	OpSNPV
Bombyx mori NPV	BmNPV	*Rachiplusia ou* MNPV	RoMNPV
Choristoneura fumiferana MNPV	CfMNPV	*Spodoptera exigua* MNPV	SeMNPV
Galleria mellonella MNPV	GmMNPV	*Spodoptera frugiperda* MNPV	SfMNPV
H. zea SNPV	HzSNPV	*Trichoplusia ni* MNPV	TnMNPV
Lymantria dispar MNPV	LdMNPV	*Trichoplusia ni* SNPV	TnSNPV
Mamestra brassicae MNPV	MbMNPV		

Source: Murphy et al. (1995)

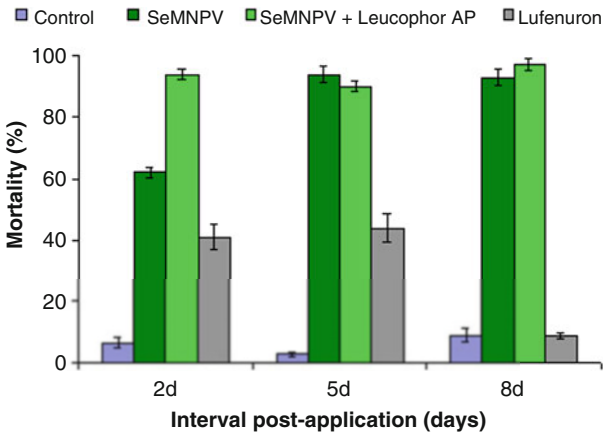

Fig. 1.6 Application of nucleopolyhedrovirus (SeMNPV) as a biopesticide against chemical insecticide in crops (Lasa et al. 2007)

and is highly stable in the protein. In the alkaline midgut, the virus particle is released and the larva is infected. Baculovirus as a biopesticide is used within the case of *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV) that is employed to manage the velvet caterpillar in soybean (*Glycine max*) crops. Instead of antibiotics, baculovirus may also be used to control the host population by causing serious and sudden outbreaks of their host populations that lead to complete control. Baculoviruses as biopesticides have several benefits as tool in IPM which comprise extreme specificity, safe to vertebrates and plants, and easy genetic manipulation against chemical insecticides (Table 1.10). However, baculoviruses face some limitations like other biopesticides, if it is used at broad spectrum in agricultural and horticultural crops (Fig. 1.7) (Possee et al. 1997; Inceoglu et al. 2006; Mills and Kean 2010; Ravensberg 2011a; Regnault-Roger 2012).

In order to be effective, in vivo mass production of baculoviruses is associated with high manufacturing costs. Upon death, that target body of insects is available as part of the continuing cycle of life and death for other larvae for consumption. Baculovirus insecticide characteristics are often enhanced by using formulations consisting of stilbene-derived optical brighteners. These compounds will improve the susceptibility to NPV infection by disrupting the peritrophic membrane or by inhibiting insect midgut cells' sloughing or virus-induced apoptosis (Washburn et al. 1998; Wang and Granados 2000; Okuno et al. 2003; Dougherty et al. 2006).

The first well-documented environmental introduction of baculovirus, which led to successful removal of the insect pest, accidentally happened before World War II (Dutta 2015). Baculovirus proved to be a useful approach to combat insect pests. As a result of this, several natural baculoviruses, extremely particular pathogen, without deadly effect on nontarget species, have been used as biopesticides (Beas-Catena

Table 1.10 Name of the commercial products of viral biopesticides and their application in agricultural and horticultural crops

Crops	Baculovirus	Commercial products	Host insect	References
Agricultural crops				
Corn	NPV	Spodopterin	*Spodoptera littoralis*	Thakore (2006)
		–	*Spodoptera frugiperda*	Escribano et al. (1999)
Cotton	NPV	Virin-HS, DOA BIO V2 Erlandson	*H. armigera*	Erlandson (2008)
		Gemstar, Biotrol, Elcar	*H. zea*	
		Spodopterin	*Spodoptera littoralis*	Thakore (2006)
		–	*Spodoptera frugiperda*	Escribano et al. (1999)
Maize	NPV	–	*Spodoptera frugiperda*	Escribano et al. (1999)
Oilseed crops	NPV	Virosoft	*Mamestra configurata*	Erlandson (2008)
Rice	NPV	–	*Spodoptera frugiperda*	Escribano et al. (1999)
Sorghum	NPV	–	*Spodoptera frugiperda*	Escribano et al. (1999)
Soybean	NPV	Baculo-soja, Baculovirus Nitral, Coopervirus SC, Multigen, and Protégé	*Anticarsia gemmatalis*	Moscadi (1999)
			H. armigera	Erlandson (2008)
Tobacco	NPV	DOA BIO V3	*Spodoptera litura*	Kamiya et al. (2004)
Horticultural crops				
Fruit crops	NPV	VPN-82, VPN-Ultra	*Spodoptera albula*	Jackson et al. (2008)
		Spod-X, Vir-ex, Spod-X LC, Otienem-STM, Ness-A, Ness-E, DOA BIO V1	*Spodoptera exigua*	Erlandson (2008)
Apple	CpGV	Capex 2	*Adoxophyes orana*	Dickler (1991)
			Cydia pomonella	Vincent and Andermatt (2007)

(continued)

Table 1.10 (continued)

Crops	Baculovirus	Commercial products	Host insect	References
Pears	CpGV	Capex 2	*Adoxophyes orana*	Dickler (1991)
			Cydia pomonella	Vincent and Andermatt (2007)
Walnut	CpGV	Cyd-X, Virosoft CP4, Madex, Granupom, Granusal, Carpovirusine, Virin-CyAp, Carposin, Carpovirus SC	*Cydia pomonella*	Vincent and Andermatt (2007)
Vegetables crops	NPV	Gemstar, Biotrol, Elcar	*H. zea*	Erlandson (2008)
		Mamestrin, Virin EKS	*Mamestra brassicae*	Thakore (2006)
		DOA BIO V3	*Spodoptera litura*	Kamiya et al. (2004)
	CpGV	Agrovir	*Agrotis segetum*	Caballero et al. (1991)
Alfalfa	NPV	Gusano biological, VPN-80TM	*Autographa californica*	Thakore (2006)
Pepper	NPV	Virin-HS, DOA BIO V2 Erlandson	*H. armigera*	Erlandson (2008)
Tomato	NPV	Virin-HS, DOA BIO V2 Erlandson	*H. armigera*	Erlandson (2008)

Notes: This list is not comprehensive because these viral biopesticides were first time described. So, readers must refer to the respective reference for more details

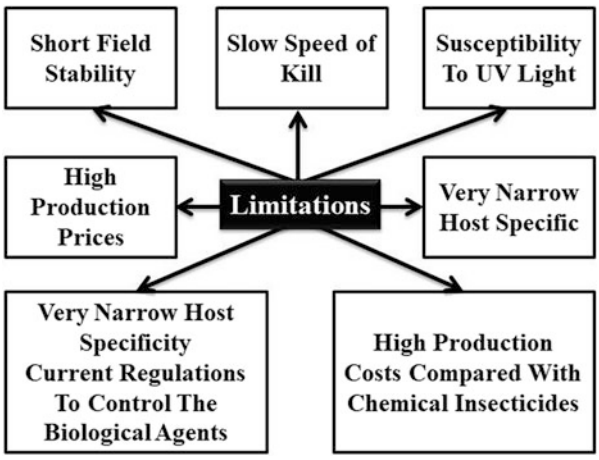

Fig. 1.7 Potential difficulties of baculoviruses as biopesticides during application in agricultural and horticultural crops (Beas-Catena et al. 2014)

et al. 2014). Natural baculovirus is used for management of pest in agricultural crops, forestry, and orchard production (Inceoglu et al. 2006).

Natural baculovirus has many benefits compared to chemical insecticides (Possee et al. 1997; Inceoglu et al. 2006). They are extremely insect-specific and strongly linked with arthropods; they are pathogenically secure for vertebrates and other useful organisms; they can be manufactured in mass, packaged, stored, and sold in a manner comparable to chemical pesticides. Around 60 pesticides based on baculoviruses were used for management of various insect pests throughout the globe (Beas-Catena et al. 2014).

Cydia pomonella granulovirus (CpGV) is another viral biopesticide, a member of the Baculoviridae family which is used to control codling moth, a pest that damages fruit trees such as apples and pears whose larvae feed on the virus during the initial phase of their growth when they come into the contact with fruit. CpGV is an extremely target-specific biopesticide which does not damage other microorganisms.

1.3.2.4 Protozoa as Biopesticide

In integrated pest management (IPM) practices, microorganisms such as protozoa are used as biopesticides (Table 1.11). They are single-celled microscopic organisms that are motile with the assistance of pseudopodia. Protozoa are used as outstanding source of biopesticides notably against several grasshopper species. However, *Nosema* spp. was the only one protozoan insecticide which has been registered in the US Environmental Protection Agency (USEPA) during the last decade. Protozoan pathogens naturally infect extensively the insect hosts. Microsporidian infections in insects are accountable for obviously the occurring low to moderate insect mortality. Some examples are *Nosema*, *Thelohania*, and *Vairimorpha*. They feed on bacteria and decaying organic matter. For example, the *Nosema locustae* protozoa have been recognized as a natural biological control agent for many grasshoppers, which infects at least 90 grasshopper species. As an example, species promising biological control are included in the *Microsporidia*. Insect microsporidian infections are considered frequent and accountable for small to mild insect mortality in natural cases. In fact, they are small creatures, which take days or decades to hurt their habitat. Instead of murdering the pest completely, they decrease host reproduction and feeding. *Microsporidia* often contagiously infect a variety of insects. There are some microsporidians that are being studied as microbial

Table 1.11 Some important examples of protozoa-based biopesticide against the targeted agents with their mode of action

Examples	Targeted agents	Mode of action
Malamoeba spp.	Locusts	When ingested, this kills insects. The feeding behavior of insects is disrupted; thus it starves and dies
Nosema spp., e.g., *N. locustae*	Grasshoppers	
Vairimorpha spp.	Lepidoptera	

insecticides. At least one is accessible on a commercial basis for 294 Current Progress in Biological Research (Hoffmann et al. 1992).

1.3.2.5 Microscopic Nematodes as Biopesticide

Nematodes are multicellular, simple roundworms rather than microbial agents. Some important species of nematodes with their host plants (viz., agricultural and horticultural) are given in Table 1.12. These products are actually used as pesticides in the same way as microbial products. However, we all are well aware of these products that can be free-living, predatory, or parasitic, almost microscopic in size, colorless, unsegmented, and have absent appendices. Many parasite species cause significant plant, animal, and human illnesses. Other species help to attack insect pests, mainly by sterilizing their hosts or otherwise weakening. Very few of them cause death of insects, yet these species are either difficult (e.g., tetradomatids) or very costly (e.g., mermithids) to generate for mass production, have small host specificities against pests of minor economic significance, have modest virulence (e.g., sphaeruliids), or otherwise are poorly suited for the purposes of pest control. In the genera *Steinernema* and *Heterorhabditis*, the only insect-parasitic nematodes with an ideal equilibrium of biological control characteristics are entomopathogenic or insecticidal nematodes. Figure 1.8 describes the mechanism of entomopathogenic nematode's action against the insects of the Lepidoptera order.

Entomogenous nematodes are generally called as those nematodes that control by infecting only insects or insect-associated arthropods (Moscardi 1999). The most commonly used species of entomogenous nematodes in formulation of insecticides are known as *Heterorhabditis heliothidis*, *Steinernema carpocapsae*, *S. feltiae*, *S. riobravis*, and *S. scapteriscae*. Sometimes, among them, the species *S. feltiae* is identified by the name of *Neoaplectana carpocapsae*. These species have different characteristics within themselves. In these characteristics, the ability of specific pests to infect and kill their various strains is prominent. There is a huge difference in their abilities, which is exhibited differently by different species. These species have been used against many pests worldwide in laboratory as well as field applications and have proved effective against over four hundred pests in the result. These insects include caterpillars, fly larvae, and many beetles (Usta 2013).

1.3.3 Biochemical Pesticides

Biochemical pesticides are naturally occurring substances whose role is mainly to control pests through nontoxic mechanisms (Fig. 1.9), whereas conventional pesticides are referred to synthetic chemicals that usually kill or inactivate insects. Biochemical pesticides contain substances that act primarily to interfere in the growth and development of insects or inhibit their sexual intercourse. For example, plant growth regulators or pheromones are those substances that act to repel or attract the pests.

Some of the important biochemical pesticides used as pest control agents are described below:

Table 1.12 Important nematode species with their host plants

Host plants	Nematode species	References
Agricultural plants		
Cotton (*Gossypium* spp.)	*Meloidogyne incognita*	Hashem and Abo-Elyousr (2011); Collange et al. (2011)
Maize (*Zea mays*)	*Meloidogyne incognita*	Hashem and Abo-Elyousr (2011); Collange et al. (2011)
Peanut (*Arachis hypogea*)	*Pratylenchus brachyurus*	Moens and Perry (2009)
Rice (*Oryza sativa*)	*Pratylenchus brachyurus*, *P. zeae*	Moens and Perry (2009)
Soybean (*Glycine max*)	*Heterodera glycines*, *Meloidogyne incognita*, *M. javanica*	Hashem and Abo-Elyousr (2011); Collange et al. (2011)
Sugarcane (*Saccharum officinarum*)	*Meloidogyne hispanica*, *Pratylenchus zeae*	Moens and Perry (2009); Maleita et al. (2012)
Tobacco (*Nicotiana tabacum*)	*Pratylenchus thornei*	Moens and Perry (2009)
Horticultural plants		
Banana and plantain (*Musa* spp.)	*Pratylenchus goodeyi*	Moens and Perry (2009)
Cassava (*Manihot esculenta*)	*Pratylenchus brachyurus*	Moens and Perry (2009)
Citrus (*Citrus* spp.)	*Pratylenchus coffeae*	Moens and Perry (2009)
Melon (*Cucumis* spp.)	*Meloidogyne hapla*, *M. incognita*, *M. javanica*	Chen et al. (2004); Collange et al. (2011); Hashem and Abo-Elyousr (2011)
Pineapple (*Ananas comosus*)	*Pratylenchus brachyurus*	Moens and Perry (2009)
Yam (*Dioscorea* spp.)	*Pratylenchus coffeae*	Moens and Perry (2009)
Broad bean (*Vicia faba*)	*Pratylenchus thornei*	Moens and Perry (2009)
Carrot (*Daucus carota*)	*Meloidogyne hapla*	Chen et al. (2004)
Eggplant (*Solanum melongena*)	*Meloidogyne hapla*, *M. incognita*, *M. javanica*	Chen et al. (2004); Collange et al. (2011); Hashem and Abo-Elyousr (2011)
Lettuce (*Lactuca sativa*)	*Meloidogyne arenaria*, *M. hapla*	Chen et al. (2004); Terefe et al. (2009)

(continued)

Table 1.12 (continued)

Host plants	Nematode species	References
Onion (*Allium cepa*)	*Meloidogyne hapla*	Chen et al. (2004)
Potato (*Solanum tuberosum*)	*Globodera pallida*, *G. rostochiensis*, *Heterodera schachtii*, *Meloidogyne hapla*, *Pratylenchus brachyurus*, *P. coffeae*, *P. penetrans*	Chen et al. (2004); Qin et al. (2000); Reitz et al. (2000); Madani et al. (2005); Moens and Perry (2009)
Sugar beet (*Beta vulgaris*)	*Heterodera schachtii*	Madani et al. (2005)
Tomato (*Solanum lycopersicum*)	*Meloidogyne arenaria*, *M. exigua*, *M. hapla*, *M. incognita*, *M. javanica*	Chen et al. (2004); Terefe et al. (2009); Rocha et al. (2010); Collange et al. (2011); Hashem and Abo-Elyousr (2011)
Tea (*Camellia sinensis*)	*Pratylenchus brachyurus*	Moens and Perry (2009)
Coffee (*Coffea* spp.)	*Meloidogyne exigua*, *Pratylenchus coffeae*	Moens and Perry (2009); Rocha et al. (2010)

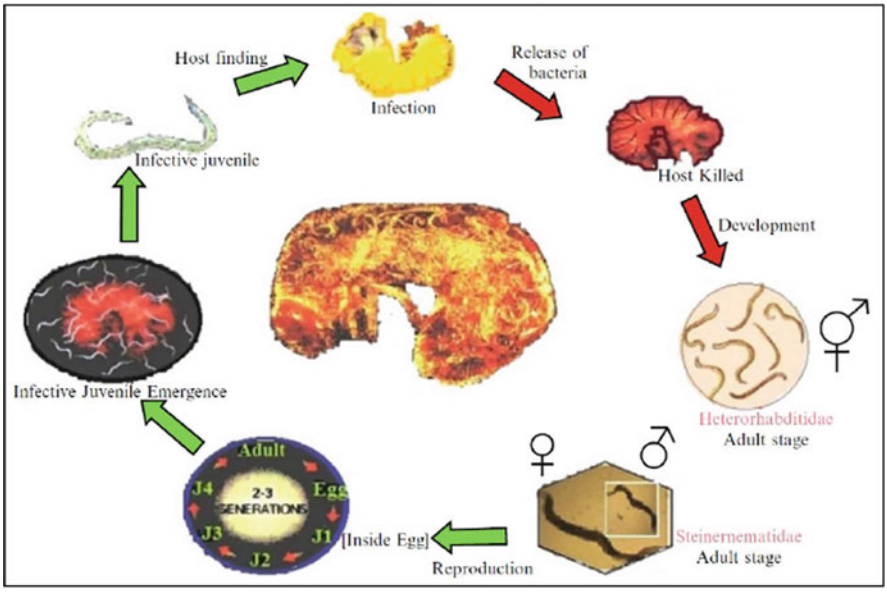

Fig. 1.8 Action mechanism of entomopathogenic nematode against insects of Lepidoptera order (Senthil-Nathan 2015)

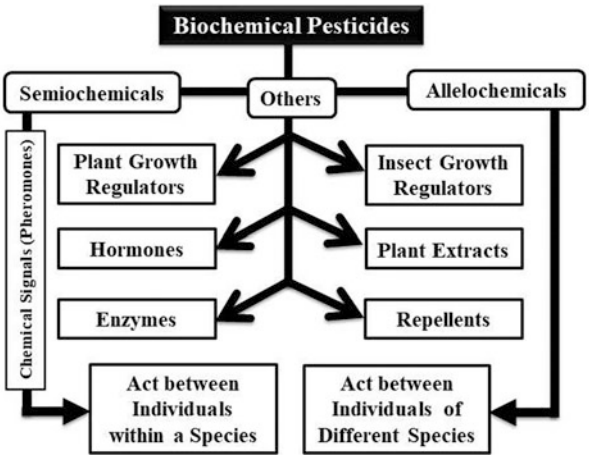

Fig. 1.9 Example of biochemical pesticides (Sarwar 2015)

1. Semiochemicals are chemical substances produced by animals or plants that change the behavior of receptor microorganisms. These include pheromones (within species) and allelochemicals (different species). The most common strategy for controlling the insects is first to attract them, second trap them, and then kill them (Norin 2007).
2. Hormones are biologically produced chemicals which are synthesized in one part of the microorganism and translocated to other parts where they control, inhibit, or regulate the numerous mechanisms.
3. Plant extracts are natural laboratories for the synthesis of a great number of chemicals. They have been used for insect control for a long time. Nicotine from tobacco (*Nicotiana tabacum*) leaves killed plum beetles; rotenone exhibits insecticidal activity. Sabadilla (*Schoenocaulon officinale*) has a major impact against caterpillars, leafhoppers, squash bugs, and stink bug (Mazid et al. 2011).
4. Enzymes are protein molecules that involve gene action expression, catalyzing biochemical reactions. The transgenic expression of insecticide proteins such as α-amylase and protease inhibitors is assessed as a prospective insect protection strategy (Schuler et al. 1998).

1.4 Status of Biopesticides in India

In sustainable agriculture, IPM is very essential for increased production and productivity. An average of Rs 200 billion annually is estimated for 33% of crop losses in India from insect pests. Furthermore, each year, Rs 1, 000 crores of agricultural exports are dismissed on the grounds that pesticide residues are inappropriate. IPM is emphasizing on the use of "bioagents in order to minimize the indiscriminate and injudicious use of chemical pesticides." Bacteria, fungi, and baculoviruses are ideal for management of the pest among the various microbial components because of their potential mass multiplication in artificial media (Vimaia

Table 1.13 Biopesticide availability in India

Biopesticides/bioagents	Approximate production (quantity/annum)
Beauveria	Meager
Bt	50,000 kg
Chrysoperla and other biocontrol insects	Meager
Lures	2 million
Neem 1500 PPM	250,000 L
Neem 300 PPM	1,000,000 L
NPV (liquid)	500,000 Le
Pheromone traps	500,000 nos.
Trichoderma	500 T
Trichogramma	1 million

Sources: Kalra and Khanuja (2007)

Table 1.14 List of registered plant- and microbe-based biopesticides in India

Name of registered biopesticide	Use for targeted agents
B. firmus	Diamondback moths
B. sphaericus	Diamondback moths
B. thuringiensis var. *galleriae*	*H. armigera*
B. thuringiensis var. *israelensis*	Diamondback moths
B. thuringiensis var. *kurstaki*	Diamondback moths
Beauveria bassiana	Mango hoppers and mealy bugs and coffee pod borer
Cymbopogon	Insect
H. bacteriophora	Borers
Neem-based biopesticides	Insect whitefly
NPV of *H. armigera*	*Helicoverpa* on chickpea
NPV of *Spodoptera litura*	*Spodoptera litura*
P. fluorescens	Bacterial and fungal pathogen
T. harzianum	Root rots and wilts
T. viride	Root rots and wilts
Trichogramma parasitoid	Sugarcane borers

Devi et al. 2012). Table 1.13 describes the annual availability of biopesticides in India.

Biopesticides (Table 1.14) are presently used in India for the management of economically important insects and pests affecting several crops of agriculture and horticulture including *B. thuringiensis* var. *kurstaki* (*Bt*k), *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Met.) Sorokin, *Paecilomyces lilacinus* Thom, *Verticillium lecanii* (Zimm.) Viegas, the entomopathogenic/nematicidal fungi, and NPVs of *H. armigera* (Hubner) and *Spodoptera litura* (Fab.). Antagonistic fungi and bacteria have been found to have potential for the management of plant disease which includes *P. fluorescens* Migula, *T. harzianum* Rifai, and *T. viride* Pers. The two major groups of entomopathogenic nematodes are

Steinernema (55 species) and *Heterorhabditis* (12 species) (Koul 2011). A commercially available biopesticide for a single main pest is Mycotal, the fungus *Verticillium lecanii*, against cereal aphids (Aquccl and Leather 2013).

1.5 Advantages and Disadvantages of Biopesticides

1.5.1 Importance and Advantages

Synthetic chemical insecticides have many advantages for food production and human health, but they also pose certain risks. There are therefore appropriate levels of pest control and fewer dangers for alternative insect management practices. The use of microbial insecticides containing microbes or their organic products is one such option. Their toxicity to the nontarget organism is exceptionally very low. Thus, microbial insecticides are particularly important. They are secure for pesticide users as well as consumers of treated plants in comparison to other frequently used insecticides. Biological pathogens and biological control agents are also known as microbial insecticides. The beneficial features of microbial pesticides were described by Jindal et al. (2013), which are given below:

1. Microbial pesticides are essentially nontoxic to nontarget organisms, communities, and human beings and do not pathogenic.
2. They have a limited range of toxicity, mostly particular for a single group or species of insect pests, and in the treated fields, they do not directly influence useful organisms (parasites, pests, pollinators, and predators).
3. The synthetic chemical insecticides can be applied together with microbial pesticides because the microbial product is not deactivated in most cases.
4. The residues of microbial pesticides do not have any adverse effects on humans and other animals; therefore, they can be used at the time of harvesting.
5. Sometimes, pathogens can be created in a pest population or its habitat and generate or seasons after seasons provide control.
6. By stimulating useful soil microflora and also increasing yield, microbial pesticides enhance root and plant growth.

1.5.2 Disadvantages

1. Since only a specific species or group of insects are toxic to a single microbial insecticide, only a portion of the pests present in the lawn, garden, or field may be controlled by each application.
2. Heat, desiccation (drying out), or exposure to ultraviolet radiation decreases the efficacy of several kinds of microbial insecticides. For some products, proper timing and implementation methods are particularly essential.
3. Certain microbial pesticides require special formulation and storage processes.

4. Most of these products are pest-specific, and therefore their prospective market is restricted. Some products are not widespread or comparatively very costly.

1.6 Summary and Conclusion

The wealthy biodiversity of India is an ace factor, offering always big sources of plant- and microbe-based biopesticide that can be used efficiently in agriculture. Indian citizens' increased health awareness has also developed a demand for organic food. This shows enormous scope for industry development of plant- and microbe-based biopesticide. With the extremely varied indigenous groups in India, the wealthy traditional knowledge base accessible can provide useful hints for the development of new and efficient plant- and microbe-based biopesticide. Of course, stress on organic farming and residue-free commodities would warrant enhanced farmers' acceptance of plant- and microbe-based biopesticide.

Demand for plant- and microbe-based biopesticide in all areas of the globe is rapidly increasing. The efficacy of plant- and microbe-based biopesticide can be equivalent to or higher than standard products when used in integrated pest management systems, particularly for plants such as fruits, vegetables, nuts, and roses. By mixing efficiency and safety, plant- and microbe-based biopesticides operate effectively, while offering flexible and minimal implementation constraints, greater possibilities for residue and strength leadership, and advantages for individual and ecosystem safety.

Plant- and microbe-based biopesticides are typically created from bacteria, fungi, viruses, protozoa, nematodes, and plant products used to manage the various types of plagues. They are particular for targets and present no or very small danger to individuals, livestock, and the world around them. The method of operation can be focused on either pest infection or physiological hunger. Instead of other chemical-based pesticides, the use of plant- and microbe-based biopesticide has resulted in healthier and secure farming methods.

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Microbial Biofertilizers and Biopesticides: Nature's Assets Fostering Sustainable Agriculture

2

Jayakumar Pathma, Rajendiran Kamaraj Kennedy, Laxman Sonawane Bhushan, Bhade Khemraj Shankar, and Kushal Thakur

Abstract

Natural products obtained from microbes, plants and animals find their potential use as biofertilizers and biopesticides sustaining and enhancing crop production and protection. Among them microbes and their metabolites with excellent plant growth-promoting and biocontrol properties have been identified, mass produced successfully, appropriately formulated and are commercially available for use. Compounds of microbial origin which make them efficient biofertilizers and biopesticides enhancing plant growth and providing protection from various biotic and abiotic stress include production of plant growth-promoting hormones like auxins, giberrelins, cytokinins and 1-aminocyclopropane-1-carboxylate deaminase (ACCD); production of antagonistic compounds such as antibiotics, crystal proteins, hydrolytic enzymes, siderophores, hydrogen cyanide, etc. Additionally, these beneficial microbes also compete for food and habitat with phytopathogens or parasitize the pests and eliminate them. Majority of the microbes and their bioactive molecules are target specific, eco-friendly and biodegradable and play an important role in preserving the ecosystem. These eco-friendly natural products could either supplement or replace the hazardous agrochemicals thereby minimize or nullify their use. Screening and selection of

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the microbial strains based on its geographical origin will make the bioformulation more suitable for a particular agroclimatic condition, and this needs intensive studies on microbial ecology and interaction with other components of the ecosystem. Research focus on ways to improve the efficacy of these biomolecules, and mass production of these natural products for its utilization and commercial availability will build a path towards environment-friendly agriculture. Alternatively, research that focusses to elucidate the chemistry of natural compounds and to synthesize compounds that mimic them is also being done to ensure the demand-supply balance. This chapter will shed light on potentials and prospectives of the use of natural bioactive compounds of microbial origin in enhancing crop protection and yield.

Keywords

Beneficial microbes · Metabolites · Biofertilizers · Biopesticides · Sustainable agriculture

2.1 Introduction

Scientific investigations record that *Homo sapiens* evolved around 250,000 BC and had a nomadic hunter-gatherer lifestyle which slowly took a change leading to the beginning of agriculture in 8000 BC. The advent of human civilization and population explosion gradually increased agricultural activities. In recent times pressurized anthropogenic activities in the name of modernization along with intensive farming employing new varieties and use of synthetic agrochemicals had not only restructured the ecosystem but also detrimentally affected the natural balance and totally looted the infinite native microfauna in the soil making it almost lifeless. The first record on the use of chemicals for insect control started as early as 2500 BC when Sumerians used sulphur for the control of insects and mites. First reports on usage of chemical fungicide, viz. lime and common salt in 1755 and copper sulphate in 1807, for control of smuts and bunts of cereals was from France. However, later many biocontrol agents were reported and used for control of insect herbivores and phytopathogens. Yet chemicals with their fast mode of action attracted the farming community and increased their dependency on agrochemicals. Chemicals were trusted to be the panacea for pest control until the first occurrence of insecticide resistance was reported for DDT against housefly in Sweden in 1946. Though problems related to pesticide resistance and resurgence started around 1950s, Rachel Carson's book *Silent Spring* in 1962 became an eye opener for the public regarding pesticide-related problems. Green revolution and introduction of high-yielding varieties demanded the use of synthetic fertilizers to fulfil the crops nutritional requirement. Conventional breeding programmes focussed on yield and quality enhancement. The high-yielding varieties showed less tolerance to biotic and abiotic stress. These factors urged the use of agrochemicals in the form of chemical fertilizers, insecticides, acaricides, fungicides, nematicides, weedicides, etc., and

their overuse had led to the hidden problem of environmental pollution by impacting soil biology and ecosystem and had started backfiring resulting in reduction of crop yield, accumulation of toxins in food and loss of ecological diversity with respect to micro- and macroflora and fauna, thereby threatening sustainability of life on earth (Edwards 1986; Kullman and Matsumura 1996; Saeki and Toyota 2004). Then came an awakening, leading to the concept of organic manures, biofertilizers, biological control (biorational biopesticides) and integrated management.

2.2 Microbes and Their Metabolites in Plant Growth Promotion

Microbes inhabiting the earth are known for their taxonomical and functional diversity. They have multiple roles in the environment and interact with other life forms in different patterns and are accordingly classified as beneficial or pathogenic or neutral. Different kinds of soil microbes especially those associated with plant rhizosphere belonging to various genera, viz. *Azotobacter*, *Azospirillum*, *Azoarcus*, *Arthrobacter*, *Anabaena*, *Bacillus*, *Burkholderia*, *Bradyrhizobium*, *Calothrix*, *Chryseobacterium*, *Delftia*, *Enterobacter*, *Frankia*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Streptomyces*, *Herbaspirillum*, *Paenibacillus*, *Pantoea*, *Phyllobacterium*, *Thiobacillus*, *Nostoc*, *Penicillium*, *Trichoderma*, *Gliocladium*, *Glomus*, *Gigaspora*, *Acaulospora*, *Scutellospora*, *Sclerocystis*, non-pathogenic strains of *Agrobacterium*, *Erwinia*, *Pythium*, *Fusarium*, *Phlebia*, *Alcaligenes*, *Chaetomium*, *Myrothecium*, *Photorhabdus* and *Piriformospora*, are known for their plant growth-promoting potential, and a few among them also act as phytopathogen antagonists and as entomopathogens (Glick 2012; Pathma and Sakthivel 2013; Sharma et al. 2013; Barman et al. 2017; Singh 2014). The microbes promote plant growth directly by synthesizing or mobilizing nutrients and by production of an array of plant growth-promoting hormones and vitamins and indirectly by protecting plants against biotic and abiotic stress (Table 2.1). Hence these could be used potentially as biofertilizers and biopesticides (Fig. 2.1).

2.2.1 Microbes Supplementing Plant Nutrition

2.2.1.1 Nitrogen

In the current scenario of intensive farming, the macronutrients, viz. nitrogen (N), phosphorous (P) and potassium (K), which play an indispensable role in plant growth are supplemented by chemical fertilizers. Though nitrogen is available in plenty in earth's atmosphere, they cannot be as such taken by plant and had to be converted into plant absorbable forms like ammonia. Agrochemicals supplementing nitrogen show a quick nitrogen release pattern resulting in a lush green growth of plants which is favourable for infection by phytopathogens and arthropod herbivores. Additionally, these nitrogenous fertilizers lead to environmental pollution, eutrophication, acid rains and ozone layer depletion. Excessive nitrogen use

Table 2.1 Microbes with PGPR traits and commercial value as biofertilizers

PGPR	Route of promotion	Beneficial host	Reference
Pseudomonas fluorescens L321	Phosphate solubilization under phosphate-limiting conditions	*Pisum sativum*	Otieno et al. (2015)
Arthrobacter protophormiae (SA3), *Dietzia natronolimnaea* (STR1) and *Bacillus subtilis* (LDR2)	Facilitate salt and drought tolerance in wheat by augmenting indole-3-acetic acid production, dropping abscisic acid/1-aminocyclopropane-1-carboxylate level and modulating expression of genes encoding for CTR1/DREB2 proteins	*Triticum aestivum*	Barnawal et al. (2017)
Enterobacter sp. NIASMVII	Production of indole acetic acid	*Triticum aestivum*	Sorty et al. (2016)
Sphingomonas sp. LK11	Alleviates salinity stress by regulating endogenous phytohormones (abscisic acid, salicylic acid and jasmonic acid)	*Solanum pimpinellifolium*	Khan et al. (2017)
Bacillus endophyticus, *Paenibacillus xylanexedens*, *Planococcus citreus*, *Planomicrobium okeanokoites*, *Sporosarcina* sp., *Staphylococcus succinus*	Direct, plant growth-promoting traits such as solubilization of phosphorus, potassium and zinc, production of phytohormones, 1-aminocyclopropane-1-carboxylate deaminase activity and nitrogen fixation, and indirect, plant growth promotion such as antagonistic production of lytic enzymes, siderophore, hydrogen cyanide and ammonia	*Triticum aestivum*	Verma et al. (2015)
P. simiae WCS417 and *P. capeferrum* WCS358	Induction of MYB72/BGLU42-dependent scopolin production and scopoletin excretion in the rhizosphere, thereby beneficial microbes increase while scopoletin-sensitive soil-borne pathogens are suppressed	*Arabidopsis thaliana*	Stringlis et al. (2018)
Trichoderma viride	Production of volatile organic compounds (VOCs) including several unknown sesquiterpenes, diterpenes and tetraterpenes which significantly increase the plant biomass, size and development of lateral roots	*Solanum lycopersicum*	Lee et al. (2016)

(continued)

Table 2.1 (continued)

PGPR	Route of promotion	Beneficial host	Reference
Dietzia natronolimnaea STR1	Alleviates salinity stress by modulation of ABA signalling, SOS pathway, ion transporters and antioxidant machinery	*Triticum aestivum*	Bharti et al. (2016)
Azospirillum sp., *A. lipoferum*,	Nitrogen fixation, phytohormone secretion	Rice, maize, wheat	de Salamone et al. (2010)
G. diazotrophicus, *Burkholderia* sp., *Herbaspirillum rubrisubalbicans*, *H. seropedicae*,	Nitrogen fixation, auxin synthesis	Sugarcane, rice, coffee, tea	Urquiaga et al. (2012)
Microbacterium sp.	Nitrogen fixation	Sugarcane	Lin et al. (2012)
Pseudomonas, *Stenotrophomonas*, *Xanthomonas*, *Acinetobacter*, *Rahnella*, *Enterobacter*, *Pantoea*, *Shinella*, *Agrobacterium* and *Achromobacter*	Nitrogen fixation, phosphorous solubilization, phytohormone production	Sugarcane	Taule et al. (2012)
Beijerinckia, *Bacillus*, *Klebsiella*, *Enterobacter*, *Azospirillum*, *Herbaspirillum* and *Gluconacetobacter*	Nitrogen fixation, phytohormone production, phosphorous solubilization	Sugarcane	Abeysingha and Weerarathne (2010)
Rhizobium sp.	Nitrogen fixation, phosphorous solubilization	Common bean and legumes	Korir et al. (2017)

impacts human health by causing methaemoglobinaemia in infants, cancer and respiratory illness (Keeney 1982). Hence judicial and need-based use of nitrogenous fertilizers along with compatible microbial counterparts which will improve the nitrogen use efficiency as well as solve the problem of nitrate accumulation in environment is essential. Biological nitrogen fixation by microbes both symbiotic and free living has been explored and has been used as alternatives supplementing plants' nitrogen requirement. Microbes belonging to genera *Rhizobium*, *Azospirillum*, *Azotobacter*, *Acetobacter*, *Bacillus*, *Cyanobacteria*, *Enterobacter*, *Frankia*, *Pseudomonas*, *Serratia* and *Streptomyces* are known to fix nitrogen as well as possess many other plant growth-promoting traits and hence had been formulated and used as biofertilizers (Bohlool et al. 1992; Kloepper and Beauchamp 1992; Hoflich et al. 1994; Cakmakci et al. 2001; Elkoca et al. 2008; Prasad et al. 2015; Bargaz et al. 2018). The microbes may exhibit symbiosis with the host plants (*Rhizobium* with legumes) or show associative symbiosis (*Azospirillum* with cereals) or be free living in soil (*Azotobacter*). These microbes produce an enzyme nitrogenase which acts as a biological catalyst and reduces atmospheric nitrogen (N_2) to

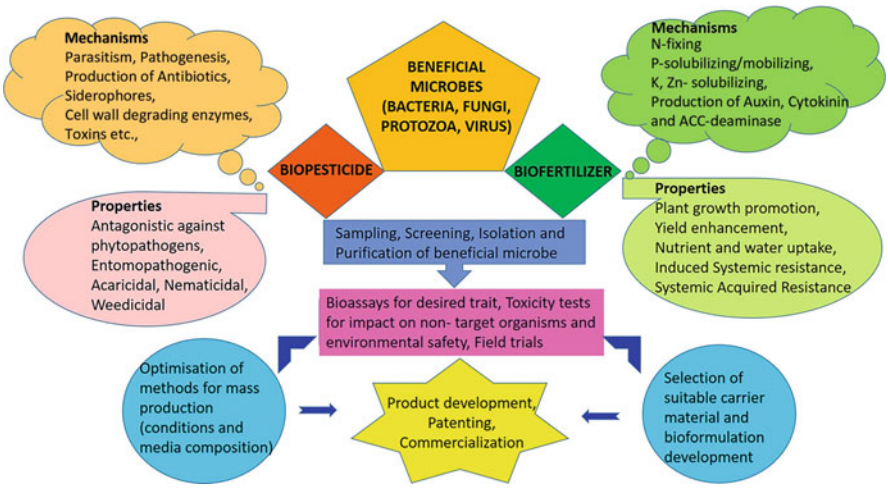

Fig. 2.1 Role of beneficial microbes in sustainable agriculture

ammonia (NH_3) through the process called biological nitrogen fixation (BNF) (Prasad et al. 2020).

2.2.1.2 Phosphorous

Soil contains huge reserves of insoluble forms of organic and inorganic phosphorous which cannot be taken up by plants. Also, the phosphate fertilizers applied get converted into forms that could not be assimilated by plants soon after application. Among several species of bacteria, fungi, actinomycetes, algae and yeasts evaluated for their phosphorous solubilizing potential, *Pseudomonas*, *Bacillus*, *Aspergillus*, *Penicillium* and *Mucor* sp. were found to be the most predominant and efficient phosphate solubilizers (Glick 1995; He et al. 1997; Illmer and Schinner 1992; Wakelin et al. 2004; Sharma et al. 2013). Other P solubilizers include *Xanthomonas* (De Freitas and Banerjee 1997), *Trichoderma* spp. (Altomare et al. 1999), *Vibrio proteolyticus*, *Xanthobacter agilis* (Vazquez et al. 2000), *Azotobacter* (Kumar et al. 2001), *Yarrowia lipolytica* (Vassilev et al. 2001), *Rhizoctonia solani* (Jacobs et al. 2002), *Rhodococcus*, *Arthrobacter*, *Serratia*, *Chryseobacterium*, *Gordonia*, *Phyllobacterium*, *Delftia* sp. (Wani et al. 2005; Chen et al. 2006), *Enterobacter*, *Pantoea*, *Klebsiella* (Chung et al. 2005), *Arthrobotrys oligospora* (Duponnois et al. 2006), *Rhizobium* spp. (Abril et al. 2007; Sridevi et al. 2007), *Kushneria sinocarni* (Zhu et al. 2011), *Schizosaccharomyces pombe*, *Pichia fermentans*, *Glomus fasciculatum*, *Paecilomyces fusisporous*, *Cephalosporium* sp., *Streptomyces* sp., *Micromonospora* sp., *Nostoc* sp., *Anabaena* sp., *Calothrix* sp., *Scytonema* sp., *Aerobacter aerogenes*, *Alcaligenes* sp., *Achromobacter* sp., *Agrobacterium* sp., *Citrobacter* sp., *Nitrosomonas* sp. and *Nitrobacter* sp. (Sharma et al. 2013). These microorganisms solubilize inorganic phosphorous by different mechanisms including production of organic (Puente et al. 2004) or inorganic acids (Azam and Memon 1996); the principle of sink theory, i.e. activate indirect P solubilization by removing and assimilating P from the liquid (Dighton and Boddy 1989); production of H_2S

(Swaby and Sperber 1958); and H^+ excretion originating from NH_4^+ assimilation (Parks et al. 1990). Organic phosphorous solubilization by production of phytases (Richardson 1994), phosphonatases and C-P lyases (Rodriguez et al. 2006), non-specific acid phosphatases (NSAPs) (Nannipieri et al. 2011), siderophores (Parker et al. 2005) and exopolysaccharides (EPSs) (Yi et al. 2008) have been reported.

2.2.1.3 Potassium

Potassium is essential for plant metabolism and is essential for activation of enzymes triggering various physiological process including plant growth and immunity. K deficiency can impact plant yield as well as increase its susceptibility to pests and diseases (Armengaud et al. 2010; Troufflard et al. 2010). In nature, potassium occurs in the soil in various plant-available, fixed forms and as minerals (biotite, feldspar, illite, muscovite, mica, orthoclase, vermiculite, smectite). The mineral deposit accounts to nearly 90–98% of total K and is not easily available to the plants. Also, the cost of K fertilizers is comparatively high and is not affordable by small and marginal farmers. Hence the use of microbes that could solubilize K from the minerals is an economical, eco-friendly alternative and is of high value. Microbes including different *Bacillus* species, viz. *B. circulans*, *B. edaphicus*, *B. mucilaginosus*, *Acidithiobacillus ferrooxidans*, *Arthrobacter* sp., *Burkholderia* sp., *Enterobacter hormaechei*, *Paenibacillus* spp., *P. mucilaginosus*, *P. frequentans*, *P. glucanolyticus*, *Aspergillus* spp., *Aminobacter*, *Sphingomonas* and *Cladosporium*, with ability to solubilize potassium, have been reported (Meena et al. 2016). The major mechanism of K solubilization by these microbes is by production of organic and inorganic acids and proton production leading to acidolysis and complexolysis and discharge of K into soil solution (Goldstein 1994; Uroz et al. 2009).

2.2.2 Microbial Metabolites Regulating Plant Growth

2.2.2.1 Auxins

"Auxin" is a phytohormone that controls the development of plant and originates from the Greek word "auxein" which means "to grow". Microbial synthesis of auxin has been reported (Patten and Glick 1996). Indole acetic acid (IAA) is reported to be the major auxin produced by microbes and is also involved in signalling in microbes influencing its gene expression and thereby influencing plant-microbe interaction (Spaepen and Vanderleyden 2011). Auxins produced by phytopathogenic bacteria, viz. *Pseudomonas savastanoi*, *Agrobacterium* spp., etc., induce tumours and galls in plants (Jameson 2000), while those produced by beneficial plant growth-promoting rhizobacteria, viz. *Pseudomonas fluorescence*, *Azospirillum*, *Bacillus* spp., etc., help in the development of plant root system which is advantageous for the plants to acquire nutrients and water from a larger area (Persello-Cartieaux et al. 2003; Pathma et al. 2010; Spaepen and Vanderleyden 2011). Apart from modifying the root architecture (shortening of root length, increase in number of lateral roots and root hairs thereby increasing the surface area of active absorption), auxins also

contributes to nodule formation in legumes, vascular bundle formation, etc., and microbially produced auxins could interfere with phytoauxin transport and thereby impact auxin homeostasis (Mathesius 2008). Camerini et al. (2008) reported that microbial auxin production increased the capacity of N fixation in root nodules. *Medicago truncatula* inoculated with a strain of *Sinorhizobium meliloti* overexpressing indole-3-acetamide (IAM) biosynthesis showed increased tolerance to several stresses especially salt stress (Bianco and Defez 2009) as well as recovered from phosphorous deficiency (Bianco and Defez 2010).

2.2.2.2 Cytokinins

Cytokinins (CKs) are adenine derivative plant phytohormones that promote mainly shoot initiation/bud formation and cell division and stimulate plant developmental progression by regulating several vital physiological factors such as leaf senescence, nutrient mobilization, apical dominance and seed germination (Hussain and Hasnain 2009; Großkinsky et al. 2016). Microorganisms were also reported for the production of cytokinins especially those PGPRs from wheat, rapeseed, soybean, lettuce and pine (Liu et al. 2013). Engineered strain of *Sinorhizobium meliloti* on cytokinin synthesis confers drought tolerance to alfalfa without affecting nodulation or nitrogen fixation (Xu et al. 2012). Kudoyarova et al. (2014) reported that cytokinin-producing bacteria (*Bacillus subtilis* IB-22) stimulates root exudation of amino acids in wheat rhizosphere and plays a vital role in enhancing rhizobacterial colonization of the rhizoplane. Recently, CK's involvement in the plant immune system has been discovered in various plants such as *Arabidopsis thaliana* (Choi et al. 2010; Argueso et al. 2012), tobacco (Großkinsky et al. 2011) and rice (Jiang et al. 2013). CK-dependent resistance is by the activation of defence responses against biotrophic pathogens such as *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*. The underlying mechanism of CK-mediated immune response towards *Pseudomonas syringae* is by the reduction of abscisic acid and induction of phytoalexin accumulation in tobacco and by stimulation of salicylic acid in *Arabidopsis* and tobacco (Großkinsky et al. 2016). Additionally, CKs are also reported for the stimulation of defence-related gene expression synergistically with salicylic acid and the accumulation of enhanced diterpenoid phytoalexin production in rice (Ko et al. 2010).

2.2.2.3 Gibberellins

Gibberellins, the plant hormone, are tetracyclic diterpenoid acid that regulates various major aspects of plant growth and development, which include seed germination, stem elongation, leaf expansion, trichome development, pollen maturation and induction of flowering (Daviere and Achard 2013). Gibberellins are also reported for the formation and maturation of legume nodulation as evidenced by mutant phenotyping, gene expression studies and transcriptional analyses of early soybean symbiotic steps (Hayashi et al. 2014). Initially, gibberellic acid was discovered in the fungus *Gibberella fujikuroi*, which causes "foolish seedling" disease in rice (Nett 2017). Though gibberellins are mostly biosynthesized in higher plants and fungus, several rhizospheric bacterial species (symbiotic and non-symbiotic) also

report for the production of variety of diterpene compounds that share common steps in the early biosynthetic pathway of gibberellins (MacMillan 2001; Bottini et al. 2004; Hayashi et al. 2014). Moreover, gibberellins have no role in fungal and bacterial metabolism; rather it is considered as secondary metabolites, which play a vital role as signalling factors between host plant and microbe (Bottini et al. 2004). Several recent studies claim that rhizobacteria-synthesized gibberellins are involved in plant growth promotion under various stress and drought conditions. Wheat roots inoculated with gibberellin-producing *Azospirillum* sp. and *Bacillus* sp. show increased nitrogen uptake compared to non-inoculants. *Azospirillum* strains increase gibberellin level in root and stimulate root growth in maize and rice seedling (Yanni et al. 2001; Bottini et al. 2004). Halo et al. (2015) demonstrated that gibberellins and an endophytic bacterium (*Sphingomonas* sp. LK11) rescued tomato plant growth and increased biomass production under salinity stress condition. *Serratia nematodiphila* PEJ1011 isolated from pepper (*Capsicum annuum*) plant rhizosphere is reported for the production of gibberellins which upholds development of plants grown under low-temperature stress (Kang et al. 2015).

2.2.2.4 Aminocyclopropane-1-carboxylate (ACC) Deaminase

ACC deaminase is an inducible enzyme, and its synthesis is stimulated in the presence of substrate ACC (ethylene precursor). The enzyme ACC deaminase converts the ACC into α-ketobutyrate and ammonia, thereby lowering the ethylene level (inhibitor of root growth) inside the plant (Glick 2005). PGPR exhibiting ACC deaminase activity has the potential to promote plant growth as well as to facilitate resistance against stressful environmental conditions including salinity, drought, flooding, metal and organic contamination and phytopathogens (Soni et al. 2018; Gupta and Pandey 2019). Maxton et al. (2018) reported that the ACC deaminase-producing *Burkholderia cepacia*, *Citrobacter freundii* and *Serratia marcescens* bacterial inoculants alleviate the effects of drought and salt stress in *Capsicum annuum* by promoting root system with increased plant biomass. Similarly, plant *Panicum maximum* inoculated with ACC deaminase-producing rhizobacteria significantly improved its membrane stability, content of proteins, phenolics and photosynthetic pigments as well as enhanced water conservation under drought and salt stress condition (Tiwari et al. 2018). The strain *Serratia* K120 exhibits positive correlation of ACC deaminase activity in the presence of heavy metals and increases the *Helianthus annuus* plant height and root length (Carlos et al. 2016). Therefore, PGPR possessing ACC deaminase activity plays an immense role in the agriculture sector in mitigating the adverse effect of biotic and abiotic stresses.

2.3 Microbial Metabolites in Pest Management

Nature provides various checks to balance the biotic components of any ecosystem. These mechanisms benefit certain living forms and act negatively on others. For example, certain microbial epiphytes and endophytes associated with plants offer protection against the invading arthropod pests and phytopathogens by activating

plant defence mechanisms, while a few microbes present in the ecosystem act as entomopathogens or as agonists to phytopathogens. Identification and use of these microbes as biopesticides will reduce the problems associated with environmental pollution, toxic residues in food and feed, pesticide resistance, etc., as these biopesticides are highly target specific, easily biodegradable and economical. Biopesticides occupy 5% of the total pesticide market globally accounting to nearly $3 billion in 2013 and are expected to increase to $4.5 billion by 2023 with microbial products taking lead (Olson 2015; Dunham 2015). Microbes are prolific producers of secondary metabolites and bioactive enzymes which are inhibitory or lethal to phytopathogens, arthropod herbivores and plant-parasitic nematodes. Availability of around 1400 biopesticide products worldwide has been reported (Marrone 2008). Survey reports that 15 microbial species as 970 different formulations are registered for use as biopesticides in India till date as compared to a developed country like the United States where 57 different microbes as 356 biopesticide formulations were registered for use by 2017 (Kumar et al. 2018; Arthurs and Dara 2018; CIB Rc 2019). In India microbial biopesticides occupy only 4% share of the total pesticide market (Singh 2014).

2.3.1 Arthropod Management

Though history of biopesticides dates back to 1800s, microbial control of insect pests starts with the discovery of *Bacillus thuringiensis* (*Bt*) in the beginning of the twentieth century. Until 1977 only one *Bt* serotype *kurstaki* with insecticidal property against lepidopterans was identified, and later serotypes *israelensis* (*Bti*) and *morrisoni* effective against dipterans and coleopterans, respectively, were discovered in 1977 and 1983 and used as biopesticides. In 1995 the first transgenic *Bt* corn was registered with the Environmental Protection Agency (EPA) for commercial cultivation (Sanahuja et al. 2011). *Bt* produces delta-endotoxins, namely, the crystal proteins (Cry), which are highly strain specific with varying degree of bio-efficacy against different insect orders. As the insect ingest the *Bt* Cry proteins, the endotoxin gets activated by the alkaline pH of the insect gut and disrupts the gut cell wall; the infected insect stops feeding and dies within 2–3 days. The acidic nature of human gut makes *Bt* safe for humans. Though environmentally safe microbial products have less shelf life and are not environmentally stable. UV degradability of *Bt* formulations before reaching the target and their high target specificity were not appreciated by farmers, and they preferred broad-spectrum chemicals with long-lasting effects that would tackle multiple pest problems. So research still continues in finding potent strains with broader target range and environmental stability. Improved fermentation and harvesting technologies, easy to use and stable formulations with effective carrier materials and UV protectants were explored. The advent of molecular biology, genomics and recombinant DNA technology provided a path of tailoring the strain biology to improve its virulence and bio-efficacy and to widen its host range. Bacterial direct transformation or simple conjugation has been used to combine multiple toxins as in the case of strain

EG2424 that produces Cry1Ac and Cry3A from *Bt kurstaki* (active against the European corn borer) and *Bt tenebrionis* (active against the Colorado potato beetle, *Leptinotarsa decemlineata*), respectively (Carlton and Gawron-Burke 1993). In cases where conjugation is unsuccessful due to plasmid incompatibility, artificial transformation by cloning genes of interest into *Escherichia coli* is adapted (Arantes and Lereclus 1991). Encapsulation of Cry proteins in bacterium *Pseudomonas fluorescens* improves their chemical and UV stability as well as yields higher amounts of Cry proteins due to the high-expression constructs; however their level of persistence in the environment is lesser than that of *Bt* spores. Similar construct using an endophytic bacterium *Clavibacter xyli* var. *cynodontis* with improved penetration into plants vascular system thereby conferring resistance to European corn borer has been developed (Lampel et al. 1994; Baum et al. 1999). Yuan et al. (1999) reported that expression of *Bacillus sphaericus* and *Bti* toxins in a single cell could increase the products larvicidal efficacy against mosquitoes. Ghaffar et al. (2008) reported a construct of *Bt* co-expressing Cry1C with Cry1Ag which reduced the LC_{50} values to 2.2 ppm in contrast to LC_{50} values of 104 ppm and 64 ppm by individual *Bt* strains expressing cry1Ag – BT4 – and those expressing Cry1C, respectively, against *Spodoptera littoralis.* Though *Bt* transgenic crops capable of producing *Bt* endotoxins with improved target specificity and environmental stability have been explored as effective alternatives to topical *Bt* applications, pronounced cumulative effects of transgenic crop on the evolutionary behaviour and health of organisms involved in the guild need extensive research over a long period. Other bacterial entomopathogens with potential to be used as biopesticides include *Bacillus (Lysinibacillus) sphaericus*, *Paenibacillus* spp., *Brevibacillus laterosporus*, *Burkholderia* spp., *Chromobacterium* spp., *Pseudomonas entomophila*, *Serratia entomophila*, *Yersinia entomophaga*, *Streptomyces* spp., *Saccharopolyspora* spp. and entomopathogenic nematode symbionts *Xenorhabdus* spp. and *Photorhabdus* spp. (Lacey et al. 2015; Ruiu 2015). Vegetative cells of *B. sphaericus* produced mosquitocidal toxins (Mtx), while endospores produced binary protein toxins (BinA and BinB) (Baumann et al. 1991; Charles et al. 2000). Formulations containing *B. sphaericus* effective against dipterans are commercially available (Ruiu 2015). *Paenibacillus popilliae* and *P. lentimorbus* cause septicaemia and milky disease in herbivorous coleopterans and could be used as a biopesticide (Zhang et al. 1997). *Brevibacillus laterosporus* was reported to be pathogenic to numerous invertebrates as well as microbial phytopathogens. Insecticidal toxins of *Bacillus laterosporus* mimic vegetative insecticidal proteins of *Bt* and has the same mode of action. It is found to effectively control corn rootworms (*Diabrotrica* spp.), mosquitoes (*Aedes aegypti)* and housefly (*Musca domestica)* (Ruiu et al. 2007; Ruiu 2013). Endosymbionts, viz. *Photorhabdus* spp. and *Xenorhabdus* spp., of entomopathogenic nematodes produce a complex of insecticidal toxin (Tc), while *Photorhabdus luminescens* also produce insect-related (Pir) proteins which have potential insecticidal activity (Ffrench-Constant and Waterfield 2006). Actinobacteria *Streptomyces* spp. produce various compounds such as antimycin A, flavensomycin, macrotetralides, macrocyclic lactones, piericidins and prasinons with insecticidal and anti-helminthic properties of which avermectins and

ivermectins, produced by *S. avermitilis*, and milbemectin, from *S. hygroscopicus* and their analogues (emamectin), have been successfully commercialized and used as effective biopesticides (Copping and Menn 2000; Ruiu 2015). Another important insecticidal compound spinosad developed from spinosyn produced by actinobacteria, viz. *Saccharopolyspora spinosa*, with broad-spectrum activity against Lepidoptera and Diptera has played an important role in pest control (Sparks et al. 2001).

Other major microbes with insecticidal properties successfully governing the biopesticide industry include entomopathogenic fungus *Metarhizium anisopliae*, *Lecanicillium lecanii*, *Beauveria bassiana*, *B. brongniartii*, *Isaria fumosorosea* and *Hirsutella thompsonii.* Most of the entomopathogenic fungi belong to order *Entomophthorales* and *Hypocreales*. *Beauveria bassiana* was the first entomopathogenic fungi to be identified to cause "white muscardine disease" in silkworm in 1835 by Agostino Bassi, the father of insect pathology, and later in 1888 Elie Metchnikoff documented the entomopathogenic potential of *Metarhizium anisopliae* which caused "green muscardine disease". Both these fungi were used for controlling economically important pests such as aphids, thrips, whiteflies, beetles, weevils, grasshoppers, butterflies, moths, termites, mosquitoes and cockroaches (Hussain et al. 2014). Majority of the mycoinsecticidal products in the world market contains *M. anisopliae* and *B. bassiana* covering 33.9% each, while *I. fumosorosea* and *B. brongniartii* formulations cover 5.8% and 4.1%, respectively. Products from *H. thompsonii* were popular as mycoacaricides. They are formulated as wettable powders, technical concentrates containing fungus-colonized substrates and oil dispersions (De Faria and Wraight 2007). Entomopathogenic viruses also play an important role in the management of insect pest especially lepidopterans. Khachatourians (1986) reported that nearly 650 entomopathogenic viruses have been isolated from insects. Among them baculoviruses with dsDNA isolated from nearly 700 different arthropod species are important group of entomopathogens with respect to effectiveness and commercial availability (Popham et al. 2016; Kergunteuil et al. 2016). Commercial availability of nearly 60 baculovirus biopesticide products has been documented around the globe. Important entomopathogens of commercial value are listed (Table 2.2).

2.3.2 Disease Management

An array of soil microbes especially those inhabiting the rhizosphere region, their metabolites and enzymes are effective in controlling phytopathogenic diseases either by their antagonistic activity or by inducing plant defences (Pathma et al. 2011; Spence et al. 2014). Majority of them belong to *Pseudomonas*, *Bacillus*, *Streptomyces* and *Trichoderma* and are abundant in the plant rhizosphere (Pathma et al. 2011; Berg 2009). These microbes exhibit an array of mechanisms, viz. competition, antagonism, mycoparasitism and induced systemic resistance to combat phytopathogens. Biocontrol potential of microbes might be due to their potential to produce secondary metabolites with antagonistic properties, viz. antibiotics

Table 2.2 Entomopathogenic microbes of commercial importance as biopesticides

Microbial species	Target organism	Reference
Entomopathogenic bacteria		
Bacillus thuringiensis ssp. *kurstaki*	Lepidopterans	Frankenhuyzen (2009)
B. thuringiensis ssp. *aizawai*	Lepidopterans	Mashtoly et al. (2011)
B. thuringiensis ssp. *japonensis*	Coleopterans	Mashtoly et al. (2010)
B. thuringiensis ssp. *israelensis*	Dipterans	Roh et al. (2007)
B. thuringiensis ssp. *tenebrionis*	Coleopterans	Roh et al. (2007)
Paenibacillus popilliae	Coleopteran (*Popillia japonica*)	Koppenhofer et al. (2012)
Serratia entomophila	Coleopteran	Jackson Lacey et al. (1992)
Entomopathogenic actinomycetes		
Saccharopolyspora spinosa	Dipteran, lepidopteran, thysanopteran, psocopteran, hemipteran, Acarina	Kirst (2010); Bacci et al. (2016)
Streptomyces spp.	Coleopterans, lepidopterans	Wang et al. (2011); Arasu et al. (2013)
Entomopathogenic fungi		
Metarhizium anisopliae	Coleopteran, dipteran, hemipteran, isopteran	Lacey et al. (2011), Jaronski and Jackson (2012)
Beauveria brongniartii	Coleoptera (Scarabaeidae)	Townsend et al. (2010)
Lecanicillium longisporum	Hemipterans	Down et al. (2009); Kim et al. (2009)
Conidiobolus thromboides	Hemipterans, thysanopterans	Hajek et al. (2012)
Aschersonia aleyrodis	Hemipterans	Lacey et al. (2011); McCoy et al. (2009)
Entomopathogenic virus		
Spodoptera litura NPV (SlNPV)	*Spodoptera litura*	Erayya et al. (2013)
Spodoptera exigua (SeNPV)	*Spodoptera exigua*	Erayya et al. (2013)
Cotton bollworm NPV (HearNPV)	*Helicoverpa armigera*	Rowley et al. (2011)
Corn earworm NPV (HezeSNPV)	*Helicoverpa zea*, *Heliothis virescens*	Rowley et al. (2011)
Velvetbean caterpillar NPV (AngeMNPV)	*Anticarsia gemmatalis*	Panazzi (2013)
Alfalfa looper NPV (AucaMNPV)	Noctuidae	Yang et al. (2012)
Tea moth (BuzuNPV)	*Buzura suppressaria*	Yang et al. (2012)
Amsacta moorei NPV	*Amsacta moorei*	Erayya et al. (2013)
Agrotis ipsilon, *A. segetum* NPV	*Agrotis ipsilon*, *A. segetum*	Erayya et al. (2013)

(continued)

Table 2.2 (continued)

Microbial species	Target organism	Reference
Trichoplusia ni NPV	*Trichoplusia ni*	Erayya et al. (2013)
Anadividia peponis NPV	*Anadividia peponis*	Erayya et al. (2013)
Lymantria dispar NPV	*Lymantria dispar*	Erayya et al. (2013)
Mamestra brassicae NPV	*Mamestra brassicae*	Erayya et al. (2013)
Neodiprion lecontei NPV	*Neodiprion lecontei*	Erayya et al. (2013)
Orgyia pseudotsugata NPV	*Orgyia pseudotsugata*	Erayya et al. (2013)
Cydia pomonella granulovirus (CpGV)	Apple codling moth	Erayya et al. (2013)
Adoxophyes orana GV	*Adoxophyes orana*	Erayya et al. (2013)
Agrotis segetum GV	*Agrotis segetum*	Erayya et al. (2013)
Diamond back moth GV	*Plutella xylostella*	Rowley et al. (2011)
Entomopathogenic protozoan		
Nosema locustae	Grasshoppers and locusts	Henry and Oma (1981)
Paranosema locustae	Grasshoppers	Lange and Cigliano (2005)
Nosema pyrausta	European corn borer	Lewis et al. (2009)
Nosema lymantriae	Gypsy moth	Solter et al. (2012)
Vairimorpha disparis	Gypsy moth	Solter et al. (2012)
Vairimorpha invictae	Fire ants	Briano et al. (2006)
Kneallhazia solenopsae	Fire ants	Briano et al. (2006)
Amblyospora connecticus	Mosquito	Solter et al. (2012)
Edhazardia aedis	Yellow fever mosquito	Andreadis (2007)

(phenazines, phloroglucinols, 2,4-diacetylphloroglucinols, agrocin 84), lipopeptides (iturin A, bacillomycins, iturins, mycosubtilin, fengycins, plipastatin), macrolactones (plipastatins, fengycins and surfactins), production of iron-chelating siderophores (pyoverdines, pyochelin, quinolobactin, ornicorrugatin, pseudobactin), extracellular hydrolytic enzymes (chitinase, β-1,3-glucanase, laminarinase), root colonization and providing a protective shield masking infection sites, detoxification of virulence factors causing phytotoxicity and acting as elicitors of inducing resistance in host plants (Pathma et al. 2011; Singh 2014; Shafi et al. 2017). Certain strains of microbes show antagonism against a wide range of phytopathogens causing leaf spots, blast, downy and powdery mildews, root rots, stem rots, damping off, wilt, scab, canker, fruit rots, etc., and such organisms effective against multiple pathogens have more value as biocontrol agents (Table 2.3). Alternatively, formulations containing microbial consortia with two or more microbes with

antagonistic activity and multiple disease-evading or tolerance mechanism have been developed to combat disease complex in crop plants. Combined use of strains exhibiting multiple biocontrol mechanisms against a pathogen or against a wide range of phytopathogens will improve the virulence and biocontrol property of the formulation, especially in cases where certain environmental factors including the soil nutrient content or plant exudates hinder the performance of the strain which performed better in other geographical locations (Kumar and Jagadeesh 2016). In spite of all these advantages, the success of microbial consortia primarily depends on their compatibility and additive and synergistic activity. Research unveil that microbial consortia could enhance transcriptional activation of many metabolic pathways which have a pronounced cascading effect on defence signalling in the host plant against a variety of phytopathogens and also certain arthropod pests. Moree et al. (2012) reported that metabolic profiling of microbial communities using microbial imaging mass spectrometry unveiled the spatio-temporal dynamics of metabolite production and metabolic transformations occurring due to microbial interplay. Thus metabolic profiling of microbes in isolation and in combination with other microbes and research on the effect of microbe or microbial community complex and associated transcriptional regulations and host physiological response will help us identify the best combinations which perform effectively under field conditions and provide good cost-benefit ratio for commercialization benefitting sustainable and profitable agriculture.

2.3.3 Nematode Management

Nematodes cause considerable loss to crop plants worldwide which is estimated to be around 12% annually (Trivedi and Malhotra 2013). Around 250 nematode species belonging to 43 genera are reported as crop pests (Chelinho et al. 2017). Numerous soil microbes with agonistic properties against these plant-parasitic nematodes (PPN) have been reported which includes bacteria, fungi and actinomycetes. Few species of fungi belonging to genera *Arthrobotrys* (*A. oligospora*, *A. superba*, *A. anchonia* and *A. dactyloides*), *Dactylaria brochopaga*, *D. candida*, *Dactylella lobata* and *Monacrosporium cionopagum* predate upon PPN by forming trapping structures such as constrictive rings, adhesive knobs, constrictive but non-adhesive rings and network traps and are referred as "predatory fungus". Fungi, viz. *Paecilomyces lilacinus*, *Pochonia chlamydosporia* and *Verticillium* spp., are documented to be efficient egg parasites of PPN. Certain fungi form adhesive spores which stick on to the nematode cuticle and infest the PPN, and the list includes *Hirsutella* sp., *Catenaria anguillulae*, *Myzocytium lenticulare*, *M. anomalum*, *Meristracum asterospermum*, *Meria coniospora* and *Nematoctonus leiosporus* (Cayrol et al. 1992). Sarhy-Bagnon et al. (2000) reported a compound 6-pentyl-α-pyrone from *Trichoderma harzianum* with antagonistic potential against PPN. Though many fungal species were found to be effective against PPN, only a few among them, viz. *A. oligospora*, *P. lilacinus*, *P. chlamydosporia* and *T. harzianum*, were successfully employed as commercial

Table 2.3 Microbial genera combating phytopathogens of commercial value as biocontrol agents

Microbe	Activity	Host plants	Target phytopathogens	Mechanism(s)	Reference
Trichoderma viride, *T. harzianum*	Biocontrol, biofertilizer, biostimulant	Cereals, pulses, oil seeds, vegetables, sugar crops, spices and condiments	*Alternaria* sp., *Fusarium* spp., *Rhizoctonia* sp., *Botryodiplodia* sp., *Macrophomina* sp., *Pythium* sp., *Botrytis* sp., *Sphaerotheca* sp., *Sclerotinia* sp.,	Induction of ISR and SAR against biotic and abiotic stresses, production of antifungal and antibacterial metabolites, siderophores, phosphorous solubilization, phytohormone production	Woo and Pepe (2018); Ghazanfar et al. (2018)
Pseudomonas fluorescens, *Pseudomonas* spp.	Biocontrol, biofertilizer, biostimulant	Rice, wheat, pea, sugar beet, potato, cucumber, radish, cotton, tobacco, groundnut, mango, banana, tea	*Fusarium* spp., *Rhizoctonia sp.*, *Macrophomina* sp., *Pythium* sp., *Botrytis* sp., *Pestalotia* sp., *Colletotrichum* sp., *Cylindrocladium* sp., *Magnaporthe* sp.	ISR, production of siderophores, antibiotics, phytohormones, cell wall-degrading and cell wall-denitrifying enzymes, competition, etc.	Pathma et al. (2010, 2011); Couillerot et al. (2009)
Bacillus subtilis, *B. megaterium*, *B. pumilus*, *B. amyloliquefaciens*, *B. cereus*, *Bacillus* spp.	Biocontrol, biofertilizer, biostimulant	Cereals, cotton, vegetables, oil seeds, mulberry, banana, avocado, ginseng, *Arabidopsis*, tobacco, cucumber, watermelon	*Fusarium* spp., *Verticillium* sp., *Colletotrichum* sp., *Ralstonia* sp., *Sclerotinia* sp., *Pythium* sp., *Phytophthora* sp., *Pseudomonas syringae*, *Septoria* sp.	Production of antibiotics, lipopeptides, antifungal proteins, cell wall-degrading enzymes, activation of plant resistance	Pathma and Sakthivel (2013); Shafi et al. (2017); Fira et al. (2018)

Streptomyces spp.	Biocontrol, biofertilizer, biostimulant	Cereals, pulses, oilseeds, vegetables, sugar crops, fibre crops, pear apple, grapevine, ornamentals	*Pyricularia* sp., *Rhizoctonia* sp., *Cercospora* spp., *Venturia* spp., *Phytophthora* sp., *Erwinia* sp., *Pseudomonas* sp., *Xanthomonas* sp., *Sphaerotheca* spp., *Botrytis* sp., *Sclerotinia* sp., *Corynespora* sp., *Cochliobolus*, *Alternaria* sp., *Helminthosporium* sp., *Uncinula* sp., *Podosphaera* spp., *Sphaerotheca* spp., *Pythium* sp., *Fusarium* sp., *Phytophthora* sp., *Rhizoctonia* sp., *Verticillium. Postia* sp., *Geotrichum* sp.	Production of antifungal and antibacterial antibiotics, secondary metabolites, fungal cell wall-degrading enzymes, etc.	Pathma and Sakthivel (2013); Aggarwal et al. (2016)

biocontrol agents (Askary and Martinelli 2015). In addition, mycorrhizal species including *Glomus fasciculatum*, in combination with *Bacillus subtilis* and *Bradyrhizobium japonicum*, and *Glomus mosseae* along with *Pseudomonas fluorescens* were reported to effectively control cyst nematode *Heterodera cajani* and root-knot nematode *Meloidogyne javanica* (Siddiqui and Mahmood 1999). Cayrol et al. (1992) documented the nematicidal effect of microbial toxin produced by *Bacillus thuringiensis* under in vitro conditions. Mateille et al. (1996) showed that the Gram-positive actinobacteria, *Pasteuria penetrans* effectively controlled many PPN especially the root-knot nematode *Meloidogyne* spp. *P. penetrans* was initially described by Throne in 1940 as a protozoan *Duboscquia penetrans*, and later it was identified as a bacterium by electron microscopic studies in 1975 (Mankau 1975). It was appropriately renamed as *Pasteuria penetrans* and was reported from nearly five continents and islands of Indian, Pacific and Atlantic oceans. It was found to parasitize nearly 208 nematode species from 96 genera of 10 different orders with many reports on control of root-knot and cyst nematodes (Sayre and Starr 1985). Rhizosphere bacteria, viz. *Agrobacterium radiobacter*, *Azotobacter chroococcum*, *Arthrobacter*, *Azotobacter*, *Aureobacterium*, *Alcaligenes* spp., *Bacillus* spp., *Beijerinckia*, *Burkholderia*, *Chromobacterium*, *Clavibacter*, *Clostridium*, *Curtobacterium*, *Comamonas*, *Corynebacterium paurometabolum*, *Desulfovibrio* spp., *Enterobacter*, *Flavobacterium*, *Hydrogenophaga*, *Gluconobacter*, *Klebsiella*, *Methylobacterium*, *Serratia* spp., *Stenotrophomonas*, *Phyllobacterium*, *Sphingobacterium*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Rhizobium* spp., *Streptomyces* spp., *Serratia,* and *Variovorax*, were reported to have potential nematicidal properties and are of great value for commercialization as a bioformulation. Mode of action includes parasitism; competition for nutrients; production of toxic secondary metabolites, antibiotics and nematode cell wall-degrading enzymes; meddling with host plant recognition by nematodes; inducing systemic resistance in plants; and supporting plant health (Siddiqui and Mahmood 1999). Rahul et al. (2014) reported the nematicidal activity of a bioactive metabolite prodigiosin present in the pigment produced by *Serratia marcescens.*

2.3.4 Weed Management

Weeds are unwanted plants that grow along with crop plants and compete with them for nutrition and other resources and can cause considerable yield loss if not addressed in the appropriate time. Nearly 30,000 weed species have been identified to cause economic damage worldwide out of which 1800 species are identified as noxious causing an average yield loss of 9.7% of total crop production annually (Li et al. 2003). Continuous use of chemical weedicides had created the problem of herbicide resistance in weeds, and nearly 32 glyphosate-resistant weed species were reported worldwide (Heap 2015). Initially many fungal species were studied for their potential as mycoherbicides including *Colletotrichum gloeosporioides* f. sp. *cuscutae* to control dodder, etc. (Gao 1992), and their fungal spore suspensions were commercially formulated which included De Vine, Stump-Out, Collego,

Biomal, Dr. BioSedge, etc. However, many factors such as mass production, optimum activity conditions, etc. hindered their potential and popularity as bioherbicides. Later bacteria especially PGPR were explored for their possibility to be used as bio-weedicides and were found to be more advantageous (Li et al. 2003). Bacteria belonging to genera *Pseudomonas* and *Xanthomonas* and fungi belonging to genera *Colletotrichum*, *Sclerotinia* and *Phoma* have been reported as potential bio-weedicides (Harding and Raizada 2015). *Xanthomonas campestris* pv. *poannua* was used for controlling *Cynodon dactylon* (Bermuda grass) and *Poa annua* (bluegrass) (Johnson 1994). Also, genetic modification to improve the virulence of *Xanthomonas campestris* pv. *campestris* using genes encoding bialaphos production was attempted (Charudattan and Dinoor 2000). Phytoxins produced by *Pseudomonas syringae* pv. *phaseolicola* inhibited *Bromus tectorum* (downy brome). Combination of *Pseudomonas putida* ATH-1RI/9 and *Acidovorax delafieldii* ATH2-2RS/1 potentially controlled *Abutilon theophrasti* (velvetleaf) by production of HCN (Owen and Zdor 2001). Different pathovars of *P. syringae*, namely, *phaseolicola*, *tabaci* and *syringae*, were reported to produce phytotoxins, viz. phaseolotoxin, tabtoxin and syringomycin, respectively, with specific mode of action such as inhibition of arginine synthesis, inhibition of glutamine synthetase and hydrolysis of cell membranes, respectively, thereby killing the target organism (Li et al. 2003). Different actinomycete species such as *Streptomyces saganonensis*, *Streptomyces actinomycetes*, *Streptomyces viridochromogenes*, *Streptomyces hygroscopicus*, etc. were reported to produce phytotoxins, viz. herbicidines and herbimycins, anismycins, bialaphos, carbocyclic coformycin and hydantocodin, respectively. Compounds such as homoalanosin, hydantocidin and phthoxazolin with potential weed control properties were also reported from *Streptomyces* (Li et al. 2003).

2.4 Challenges in Success of Microbial Bioformulation

Plant growth-promoting and biocontrol microbes and their bioactive products have potential value as biofertilizers and biopesticides which will sustain agricultural production in an economic and eco-friendly manner (Giri et al. 2019). However, lot of challenges needs to be addressed for preserving their efficacy under storage and field conditions so as to make the formulation a commercial success. Environmental factors including temperature, humidity and soil moisture play an important role in the successful establishment of the microbial bioformulation be it an insecticide, acaricide, nematicide, fungicide, weedicide or a plant growth promoter. Also, the time of application of the bioformulation with respect to the biostage of the target organisms is a crucial factor for the success of the bioagent in enhancing crop production and protection. In case of bioherbicides, increase in humidity decreases the rate of evaporation and prolongs the leaf wetness caused by spray application which in turn helps in successful colonization of the microbes (Casella et al. 2010). Different microbes will have different optimum temperature and conditions which will enhance its efficacy of performance. Both physiological and ecological considerations are essential for the success of the biocontrol programme. Thus, in

addition to laboratory and greenhouse experiments, repeated field trials will enable us to track the optimum growth conditions and their interactive effect under natural conditions. This knowledge will help to improve the efficacy and success of the commercial formulation. Another major issue is that a microbial isolate with potential biofertilizing and biocontrol properties obtained from one geographical location may not perform in a similar fashion in the other geographical limit or agroclimatic region. This is because the organism evolved in a particular region would have accustomed the environment and expressed specific traits depending on the climatic conditions, soil physio-chemistry and biology. Also the interplay between the members of the native soil microbial community and the beneficial microbe introduced as soil inoculant or through seed treatment is another crucial factor that decides the success of the microbial formulation. Isolation of microbes and their utilization in the region-specific manner could increase the success of the biocontrol programme. Apart from strain selection, preserving the viability of microbial inoculants subjected to long-term storage as well as exposed to harsh environmental conditions which would impose stress on the microbial cells is another important aspect that needs focus. Shelf life of the bioformulation is an essential criterion which will decide the success of the bioformulation and consumer preference. A shelf life of a minimum 6 months is required which will improve the confidence of the consumers to invest in bioformulations rather than in chemicals which have a very long shelf life. Also, carrier material used in the commercial product decides the product consistency and shelf life. History starts with use of soil a carrier material for rhizobium (Madhok 1934), and this concept was adapted from the farmers traditional practice of adding soil from a field cultivated with legume to a new field put under legume cultivation probably to transfer the rhizobium inoculum (Brahmaprakash and Sahu 2012). Peat followed soil in the list of carrier material used (Bashan 1998), and this is followed by lignite (Kandaswamy and Prasad 1971), coir dust (Iswaran 1972), farm yard manure (FYM) and tank silt (Bajpai et al. 1978), coal (Dube et al. 1980), fly ash (Khan et al. 2007), etc. Solid formulations in the form of granules, wettable powders, etc. were used initially but had their own drawbacks such as loss of viability due to desiccation, etc., which intensified the search for new technologies to enhance the shelf life and bioactivity of the microbial products. Other frequently used carrier materials included organic materials, such as animal manure, compost, vermicompost, sludge, sawdust, grape or sugarcane bagasse, wheat bran, oats, soy, etc., and inert materials such as silicates, vermiculite, perlite, bentonite, kaolin, talc and polymers. Moisture-retaining carrier materials, viz. calcium alginate, vermiculite or oils, are known to improve the efficacy of bioformulation especially bioherbicides (Auld et al. 2003). Polymer-entrapped inoculants with better quality were introduced. Polymers were found to be efficient carriers of bacterial cells. The microbial cells are mixed with suitable polymers and subjected to chemical solidification. The cells entrapped in the polymers is then allowed to grow and then dried. Once the formulation is applied in the soil, the soil microbes degrade the polymer and thereby release the beneficial microbe in the rhizosphere which interacts with the targeted host plant and performs their function (Deaker et al. 2004). Many liquid formulations containing aqueous or oil-based

suspensions of microbial cell concentrates in the form of slurries or emulsions added with nutrients, osmo-protectants, stabilizers and adhesives with improved shelf life were introduced (Singleton et al. 2002; Malusa et al. 2012; Bashan et al. 2014). Ways to protect the introduced microbe into the new environment and preconditioning them to survive the stress imposed by the exotic environment need research focus. Focus to evaluate potent drying methods other than air-drying such as spray-drying, freeze-drying, vacuum-drying and fluidized bed drying and preconditioning of the bacterial strains, use of protectants, prompting secretion of exopolysaccharides and addition of helper strains could reduce loss in cell viability of non-sporulating microbe and improve its establishment and bio-efficacy (Berninger et al. 2018). Sahu et al. (2013) reported that fluid bed drying (FBD) increases the survival of desired microbe and reduces rate of contamination of the bioformulation.

2.5 Conclusion and Future Prospective

Knowledge on ecological interactions of the organism introduced as bioformulation and research emphasis on selection and use of microbes in the same geographic location instead of introducing the microbe isolated from a new agroclimatic zone will improve the ecological fitness of the microbe in terms of adaptability, establishment, self-perpetuation and efficacy of performance which requires repeated research trials. Development of bioformulations containing a consortium of beneficial microbes that serve multiple purpose and act as biofertilizer, biostimulant, bio-enhancer of plant resistance and biocontrol agent may act as a panacea providing a holistic plant protection and growth enhancement and needs research focus. Extensive studies at molecular level on the interactive effect of members used in the microbial consortium with regard to their compatibility, synergistic ability, etc. need to be carried out. Also, molecular studies focussing on the gene expression and protein profiling of the host plant or the target organism (pest/phytopathogen) subjected to the microbial bioformulation will provide us better insights on the mechanisms involved in plant growth promotion or pest or disease control which will help us improve the competence of the bio-inoculants. Investigations on the carrier materials, stickers, emulsifying agents and osmo-protectants used, optimum moisture and temperature requirements as well as packing material used will enhance the purity and viability, thereby improving the shelf life of the formulation which is a major factor deciding the success of commercialization and usage.

Quality products, easy to store and use formulations, efficient product distribution systems and strict regulations with regard to manufacturing and marketing of biopesticides and biofertilizers are key factors that build the base of the pyramid. Knowledge transfer to the farmers and their skill development through proper training, demonstrations and support systems to create awareness among the farmers about the sustainable effects of use of biopesticides and biofertilizers will help them reap good yield in a sustainable way which will improve their faith in organic

farming as well as attract more farming community towards using nature's assets to foster sustainable agriculture and preserve mother earth and restore its beauty.

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Microbial Factories for Biofuel Production: Current Trends and Future Prospects

3

Evangeline Christina and Valarmathi Rajendran

Abstract

The rapid depletion of fossil fuels and the increasingly growing global energy demand have paved way to find an alternative energy resource to sustain the energy crisis. Finding a cheaper and an efficient alternative energy resource could presumably be an effective method to minimize the usage of conventional fossil fuels and to combat the problem of increased greenhouse gas deposition which has adverse effects on global climate change. Directly or indirectly microorganisms play an inevitable role in the production of biofuels. The production of biofuels like bioethanol, biodiesel, biogas, and biohydrogen relies on the undeniable involvement of microbes for the conversion of a suitable substrate into a valuable biofuel. Metabolic engineering of microbes is the future of the next-generation biofuel production, which undoubtedly can lead to the engineering of superior-quality biofuel-producing microbial strains. This chapter focuses mainly on the exploitation of microbes as factories for efficient biofuel production and metabolic engineering as an effective tool to qualitatively and quantitatively increase the biofuel yield.

Keywords

Bioethanol · Biobutanol · Biodiesel · Biogas · Bioelectricity · Biomethane · Biohydrogen · Biofuel · Metabolic engineering

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3.1 Introduction

In the modern times, when mankind is standing on the edge of technological enhancement and preserving the future generations, availability of resources gives a boost to the sharpness of the edge. The ever-raging demand for a sustainable energy source can be considered as one of the major issues in the twenty-first century. Sustainable energy resources are those energy equivalents which can suffice today as well as can be preserved for the future generations. A better approach in this field can be considered as solar, wind, geothermal, and biofuel technologies, which are commendably safer but are costly. Toward these issues, significant investments are being made in the sectors, promising significant advancement in the field of sustainability.

Biofuel or bioenergy is the type of energy that is majorly produced by biological materials, specifically from microbes and photosynthetic organisms such as green plants, grasses, etc. They are sustainable, modulable according to their source, and biodegradable as well. Understanding the molecular mechanism of microbes, different approaches, such as proteomics, transcriptomics, metabolomics, fluxomics, etc., will help us identify and optimize different conditions to increase their yield (Ndimba et al. 2013), so that a better amount of biofuel can be produced. The life cycle of biofuels is depicted in Fig. 3.1. This chapter summarizes the role of various microbes that are used as cell factories for production of biofuels. Additionally, the usage of metabolic engineering and synthetic biology tools for enhanced production of biofuels has been discussed.

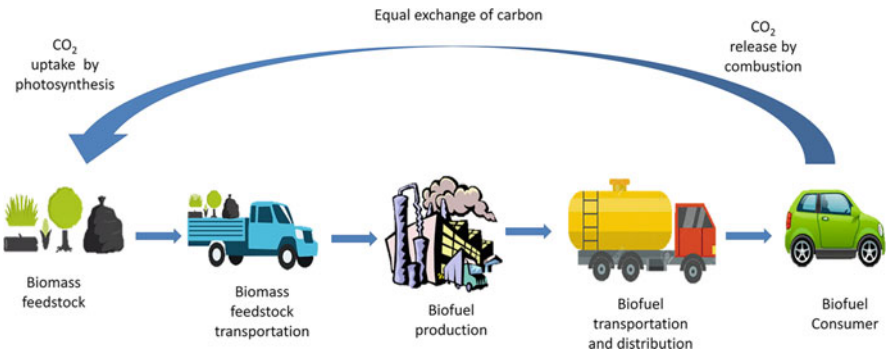

Fig. 3.1 The biofuel life cycle—carbon taken up by biomass to its emission by the end user

3.2 Need for Biofuels

3.2.1 To Combat Climate Change

Increasing concentrations of greenhouse gases (GHG) and subsequent climate change have forced humankind to search for low carbon energy resources and fuels as able alternatives. Biofuels have received tremendous attention in the recent times especially in the transportation sector where they prove their undeniable role in potentially reducing the emission of GHGs from the environment. Biofuels emit greenhouse gases significantly in a lesser quantity than the fossil fuels, which ultimately addresses the problem of carbon emission. Emission of carcinogenic compounds is considered to be reduced as high as 85% in biofuels.

3.2.2 To Build Economic Development

The economy of a country undoubtedly develops with the increase in investment on biofuels. This directly or indirectly increases job opportunities and creates new sources for income, thereby contributing to economic development in developing countries.

3.2.3 To Provide Energy Security

By 2050, the population of the world may be expected to increase to about 8–10.5 billion. With significant increase in economic growth, a substantial increase in energy consumption may also be expected. To address the growing global demand, the existing and available natural resources need to be used efficiently to ensure energy security.

3.2.4 To Provide Energy Balance

The energy balance may be defined as the ratio of the amount of energy required to produce and distribute the biofuel to the amount of energy released when it is consumed. In a scenario, where energy security is a topic of concern, biofuels are proved to have a high-energy balance in comparison with other existing fuel alternates, thereby improving energy security.

3.2.5 Biofuels Are Biodegradable and Recyclable

As biofuels are derived from natural resources, they are less likely to cause any potential harm to the environment. In comparison with petroleum, biofuels are easy and safer to handle owing to their lesser volatility. However, it can be dangerous in

case of an accidental ignition, where the fuel has inherent high energy to produce vapors sufficient enough to be ignited. Biofuels may be produced from a variety of oils and fats including waste cooking oils. This provides a chance to increase the market value of the recycled oils and also enables the biofuels to be cost-effective.

3.3 Types of Biofuels

3.3.1 Bioethanol

Conventional method of ethanol production uses food crops such as sugarcane and corn which also put a stress on production of such crops. This has already led to deforestation for cultivation of these crops which is not a feasible option. Conventionally, the lignocellulosic biomass is subjected to pretreatment with hot water, steam, acids, alkali, or even by mechanical means. Microbes such as *Aspergillus terreus*, *Trichoderma viride*, *Ceriporiopsis subvermispora*, *Pleurotus ostreatus*, *Fusarium concolor*, etc. may be used to pretreat various feedstocks such as sugarcane trash, corn stover, rice hull, wheat straw, etc. The pretreatment is intended to reduce the degree of polymerization and recover maximum quantity of lignin. The next step in the process is microbial fermentation of the pretreated feedstock. Bacteria such as *Aerobacter*, *Bacillus*, *Klebsiella*, *Thermoanaerobacter*, and *Aeromonas*; yeasts like *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus*; and fungi, namely, *Neurospora*, *Monilia*, *Fusarium*, *Mucor*, *Rhizopus*, etc., efficiently convert xylose to ethanol (Robak and Balcerek 2018). The ethanol thus formed is purified using techniques such as distillation, rectification of the distillate, and dehydration to obtain good-quality bioethanol (Jambo et al. 2016).

Lignocellulosic materials serve as an effective source of feedstock. Despite physical pretreatment methods, fungi like brown rot, white rot, and soft rot fungi can be employed from breakdown of the complex substances to a simpler form. Microbes such as *Pichia stipites* and *Candida shehatae* (Sarkar et al. 2012), thermotolerant facultative anaerobic yeast named *Kluyveromyces marxianus*, recombinant mesophilic semi-anaerobic bacteria *E. coli* strain FBR5 (Talebnia et al. 2010), unicellular cyanobacterium *Synechococcus* sp. (Deng and Coleman 1999), and genetically manipulated microbes like *Thermoanaerobacterium saccharolyticum* M0355, *Thermoanaerobacterium saccharolyticum* M1051, and *Geobacillus thermoglucosidasius* TM242 show very high yield of ethanol. Engineered bacterial strains containing genes of *Z. mobilis*, like *Erwinia chrysanthemi* (pZM15), *Klebsiella planticola* (pZM15), *Erwinia chrysanthemi* EC16 (pLOI555), and *Erwinia carotovora* SR38 (pLOI555), can be used to produce bioethanol (Ingram et al. 1998). Genetically engineered *E. coli* strains like *E. coli* K011 and *E. coli* SL40 can produce good amount of ethanol from pretreated corn fiber hydrolyzates (Bothast et al. 1999).

Microbes such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* undergo Embden-Meyerhof and Entner-Doudoroff pathway, respectively, for fermentation of sugars to bioethanol (Yang et al. 2016). Genetically engineered *E. coli* can be used

for alcohol production from hexose and pentose sugars. Coculture of *Bacillus cereus* and *Bacillus thuringiensis* produces bioethanol by simultaneous saccharification and co-fermentation (SSCF) of steam-exploded bagasse. As a result, this alleviates the pressure on food crops being used as a feedstock for bioethanol production (Ire et al. 2016).

As an alternate strategy, cellulose from newspapers can also be used as a cheaper feedstock for ethanol production. The old newspapers may be subjected to pretreatment and hydrolysis followed by fermentation. Usage of bacteria *Cytophaga hutchinsonii* for the cellulose conversion to sugar and subsequent fermentation by the yeast *Saccharomyces cerevisiae* to form ethanol have been reported (Chandran et al. 2018). Thermophilic bacteria isolated from hot springs can also be used in fermentation of lignocellulosic materials at high temperatures to produce Bioethanol.

3.3.2 Biobutanol

Anaerobic respiration is considered to be one of the oldest methods to obtain butanol on an industrial scale. Butanol is a flammable, colorless alcohol which is used as a solvent (Dürre and Ann 2008; Fortman et al. 2008). By the production of biobutanol, oil and natural gas consumption by the automobiles and thereby their harmful emissions into the atmosphere can be reduced. Therefore, butanol can effectively be used as an alternative biofuel.

Isolation and identification of an efficient butanol-producing strain and selection of a suitable substrate are the major concerns of biobutanol production. Biobutanol is reported to be produced by some species of *Clostridium acetobutylicum* through acetone-butanol-ethanol (ABE) fermentation (Dürre and Ann 2008; Qureshi and Maddox 1995). As *C. acetobutylicum* is an amylolytic bacteria, it utilizes starch as a good substrate for butanol production.

Due to high cost, generally, fermentation products are generally not used. However biobutanol is produced mainly from agricultural wastes. Substrates like waste glycerol, monosaccharides, polysaccharides, and algal biomass can be used for biobutanol production. Genetic modifications in bacteria such as *C. acetobutylicum* and *C. beijerinckii* have also shown to increase their ability to concentrate butanol (Kaminski et al. 2011).

The traditional process of distillation is used for the recovery of biobutanol. Since the boiling point of butanol is higher than that of water, the energy consumption is also higher which in turn increases the cost of the recovery process. Hence distillation lacks energetic and economic feasibility (Evasn and Wang 1988; Roffler et al. 1988). Therefore, various other cost-effective and efficient methods like adsorption, gas stripping, membrane reactors, pervaporation, etc. are being adopted for maximum recovery of biobutanol.

3.3.3 Biomethanol

The majority of petroleum fuels, namely, gasoline, diesel, LPG, CNG, etc., is consumed by the transport sector. This dependence is likely to be affected in the near future by factors such as the hike in petroleum prices, limited petroleum reserves, and the increase in number of petroleum-based vehicles worldwide. To address these issues, the search for sustainable and eco-friendly, alternative fuels is in the increasing trend.

Methanol, which can also be termed as wood alcohol, is currently made from synthetic gas and is used for fueling internal combustion engines. When synthetic gas is added with hydrogen from external sources, all of the biomass carbon gets converted into methanol carbon (Phillips et al. 1990). However, the methanol production is a cost-intensive process, and therefore it is currently made only from waste biomass, thereby ensuring an inexhaustible and surplus supply of feedstock to produce methanol (Vasudevan et al. 2005). Upon pyrolysis of wood, the resulting pyroligneous acid comprises of methanol to about 50% and other components like water, phenols, and acetone (Demirbas and Gulu 1998; Güllü and Demirbas 2001).

Methanol produced from coal is considered to be one of the most important liquid fuels of the future. Being sulfur free and devoid of other impurities, methanol from coal could effectively replace conventional motor fuels (Demirbas 2005). Using partial oxidation reactions, methanol can also be produced from natural gas (Demirbas and Gulu 1998).

Conversion of methane to biomethanol using mixed and axenic strains of *ammonia-oxidizing bacteria (AOB)* has been demonstrated (Hyman and Wood 1983; Wang et al. 2010; Taher and Chandran 2013). The methane conversion is performed by ammonia monooxygenase enzyme. The methanol produced in *methane-oxidizing bacteria (MOB)* are further acted upon and oxidized by methanol dehydrogenase (MDH) (Hanson and Hanson 1996). To prevent any further oxidation of methanol, MDH inhibitors may be added so that selective oxidation is achieved (Ge et al. 2014). However, in AOB strains, no methanol-metabolizing enzymes have been reported, thereby enabling AOB bacteria as suitable candidates for biomethanol production (Chain et al. 2003; Stein et al. 2007).

3.3.4 Biodiesel

Biodiesel is an alternative fuel to the conventional diesel and is obtained from animal fats and vegetable oils. Biodiesel is renewable, biodegradable, nontoxic, and environmentally friend and therefore has attracted worldwide attention (Xing et al. 2013). Using vegetable oils for frying generates a significant amount of used oil which may pose disposal problems. Waste oil being cheap is advantageous for biodiesel production. Triglycerides are the major constituent of vegetable oil and animal fats, and their conversion to fatty acid methyl esters yields biodiesel (Thirumarimurugan et al. 2012; Fadhil et al. 2012; Abdullah et al. 2013).

A wide variety of nonedible oils from neem, *Jatropha*, *Pongamia*, cottonseed, linseed, rubber seed, jojoba, etc. can be used as feedstock for biodiesel production. Fatty acids from animal fats and waste cooking oils also serve as excellent feedstock sources for biodiesel production (Ogunwole 2012; Gude et al. 2012). Various processes such as direct use, blending, microemulsions, and pyrolysis and transesterification are being conventionally employed in biodiesel production (Shahid et al. 2012). Biodiesel is usually blended with diesel to minimize the net CO_2 emission (Ghaly et al. 2010).

Oleaginous microbes are organisms that can store lipids up to 20% of its dry weight. But under nitrogen-limited conditions, this ability may increase and they store up to 70% (Meng et al. 2009; Rossi et al. 2011). Many filamentous fungi and yeasts are considered to be oleaginous in nature, since they have an ability to accumulate triglycerides. Actinobacterial members such as *Rhodococcus*, *Mycobacterium*, *Nocardia*, *Strepromyces*, etc. and yeasts such as *Cryptococcus* sp., *Trichosporon* sp., *Sporidiobolus* sp., *Rhodosporidium* sp., *Candida* sp., *Yarrowia lipolytica*, etc. are reported to produce and accumulate lipids (Alvarez and Steinbuchel 2002).

The extraction of lipids from the oleaginous microbes is generally carried out by a single-step solvent extraction process, a two-step solvent extraction process, or a prior mechanical treatment to these extraction processes. This involves continuous lipid extraction and solubilization of the lipids in specific organic solvents, followed by the non-lipid contaminant removal resulting in the desired final product. A single-step transesterification process can also be carried out by applying methanol and alkali such as sodium hydroxide, ammonium hydroxide, calcium oxide, zeolite, etc.; acids such as sulfonic acid, sulfuric acid, etc.; or enzymes such as *Rhizopus oryzae* lipase, *Candida antarctica* lipase, etc. to the oleaginous biomass (Niu et al. 2013; Lohit et al. 2017).

Life cycle assessment of biodiesel production by oleaginous microbes has reported that the cost of the substrates and lipid extraction process highly hinders their commercial viability at the industrial scale despite its merits (Lardon et al. 2009). However, to overcome these issues, alternative substrates that are easily available, cheap, and abundant need to be explored. Technologies to enhance efficient lipid extraction or development of an integrated biorefinery process may aid in extraction of commercially valuable compounds such as leaned biomass, flavonoids, pigments, etc. which also helps in cost management of the overall process.

By utilizing inexpensive feedstocks, oleaginous microbes reduce the cost of the biodiesel production. Adoption of eco-friendly techniques such as using green solvents in extraction process can help in valorization of the derived products which will aid further cost reduction.

3.3.5 Biomethane

The need for alternative and sustainable energy has led to the emergence of biogas, which is a versatile biofuel that can produce energy continuously and can be easily transported via the conventional gas transportation system (Mamun and Torii 2017). Biogas is a mixture of 60–70% methane and 30–40% CO_2 all together with traces of certain other gases like oxygen, nitrogen, etc. Methane is the most cleanly combustible among the other gases and produces very less soot and hence can be used as clean fuel.

Biomethane is a renewable/sustainable natural gas or biogas variant that has a higher concentration of methane proportions than with the rest of the other gas compositions, i.e., a concentration of 90% or greater (Mamun and Torii 2017). Anaerobic digestion is an extensively known process where the organic-content-rich wastewater is degraded into simpler compounds, releasing high amounts of methane. Anaerobic digestion of organic matter such as fodder, animal waste, kitchen waste, etc. can yield methane. Since anaerobic digestion can be considered as means to control biological/organic waste, it provides a window to reduce pollution as well as be a source for good biofuel (Tilche and Galatola 2008). Addition of hydrogenotrophic archaea into an anaerobic digester with biogas containing methane (60%), carbon dioxide (40%), and some external hydrogen source aids in concentrating and saturating methane from CO_2 and converts it into biomethane (Díaz et al. 2015). In an industrial scale, a hollow fiber membrane bioreactor is used which infuses 95% H_2 and CO_2 to form CH_4.

Since, biomethane is a very good alternative for conventional fuels, their application in the field of automobiles is vast. Biomethane upgraded in the form of natural gas is injected in the conventional gas grids throughout the different countries in the world. As natural gas and biomethane are intermixable, their transport through a single conventional grid is thus economy efficient and is environment-friendly as well (Svensson 2013).

3.3.6 Biohydrogen

The exhaustion of fossil fuels and subsequent global climate change have led researchers to consider using biohydrogen as an effective alternative source of energy as its consumption results only in water (Lawier and Bill 1995; Lee and Greenbaum 1995). It is also identified that the process carried out by photosynthetic bacteria favors hydrogen production from carbohydrate substrates than the non-photosynthetic bacteria (Beneman 1996).

Hydrogen is produced efficiently by various categories of bacteria. Anaerobes such as *C. pasteurianum*, *C. beijerinckii*, and *C. butyricum*; methylotrophs such as *Methylomonas albus* BG8 and *Methylosinus trichosporium* OB3b, *Pseudomonas methylica*, and *Methanothrix soehngenii*; rumen bacteria, namely, *Ruminococcus albus*; Archaea, namely, *Pyrococcus furiosus*; facultative anaerobes such as *Escherichia coli*, *Enterobacter* sp., and *Aerogenes* sp.; aerobes such as *Alcaligenes*

eutrophus and *Bacillus licheniformis*; photosynthetic purple sulfur bacteria *Thiocapsa* and *Chromatium* and non-sulfur *Rhodospirillum* and *Rhodopseudomonas*; and cyanobacteria such as *Oscillatoria limnetica* and *Anabaena cylindrica* are noteworthy of producing biohydrogen (Nandi and Sengupta 1998).

Hydrogen production by various microbes is directly linked to their energy metabolism. The mechanism of hydrogenase activity in the hydrogen-producing organisms helps dispose the electrons in excess. In aerobes, the electrons released as a result of oxidation of the substrate are transferred to the terminal oxidant which is oxygen, whereas in anaerobes, the catabolism releases electrons which use various terminal oxidants such as sulfate, nitrate, and many other organic compounds from the carbohydrate source.

Four categories of hydrogen-producing microbes have been suggested by Gray and Gest 1965:

Group 1: Strict anaerobic heterotrophs without a cytochrome system, e.g., *Clostridia* sp.
Group 2: Facultative anaerobic heterotrophs with a cytochrome system
Group 3: Strict anaerobe with a cytochrome system, e.g., *Desulfovibrio desulfuricans*
Group 4: Photosynthetic bacteria which produce hydrogen and are light dependent

Biohydrogen produced by various processes is clean, inexhaustible, and nontoxic and has high fuel efficiency. But the high cost in production, transportation, and difficulty in storage along with its highly inflammable nature limit the usage of biohydrogen. Companies that commercialize hydrogen employ other nonrenewable resources such as natural gas, oil, and coal to split hydrogen and oxygen which also significantly limits the use of hydrogen as a sustainable energy form.

3.3.7 Bioelectricity

Bioelectricity is produced by microorganisms acting on organic substrates and anaerobically digesting them. Microbes utilize the complex carbon sources as substrates, and upon oxidation it gets converted to chemical energy (Aelterman et al. 2008; Lovley 2008). Microbial fuel cells (MFCs) convert this chemical energy into electrical energy, thereby enabling sustainable production of energy.

Geobacter and *Shewanella* (Rotaru et al. 2011; Watson and Logan 2010) are the most frequently used microbes for MFC technique. Cyanobacteria such as *Anabaena* and *Nostoc* have been reported to be used as biocatalysts in microbial fuel cells (Yagishita et al. 1997, 1998). Bacteria such as *Pseudomonas aeruginosa*, *Clostridium* sp., and *Ochrobactrum pseudogrignonense* account for the predominant group which generates significant bioelectricity (Zhao et al. 2012).

Microbial metabolism releases electrons which are captured, thus maintaining a constant power density. This process occurs without considerable emission of carbon into the ecosystem. The effectiveness of the MFC unit is evaluated by multiple parameters such as COD removal rate, coulombic efficiency, and maximum

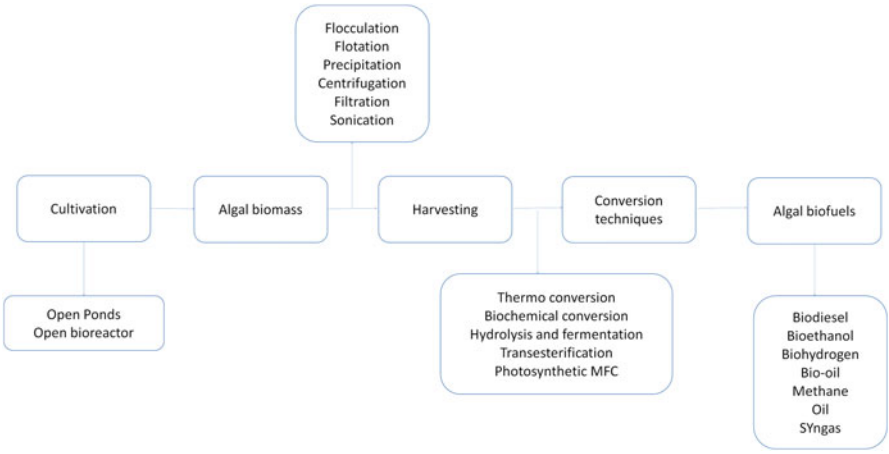

Fig. 3.2 The scheme of bioelectricity production in a microbial fuel cell

power density of power generation (Choi and Ahn 2013; Zuo et al. 2008; Min et al. 2005). A schematic representation of bioelectricity production by MFC is given in Fig. 3.2. Application of MFC technology is not limited to electricity generation. By coupling it with bioremediation strategies, MFC can prove to be an efficient method for management of municipal, industrial, and agricultural wastes (Feng et al. 2008). The power generated by microbial fuel cells was initially very low; however, due to modifications in designing, components and functioning of the apparatus have led to enhancement of the power output to an appreciable level (Li et al. 2008). This attribute enables MFCs to be applied in management and treatment of wastewater, bioremediation, and as biosensors. Table 3.1 summarizes the various organisms and the biofuel they produce from various substrates.

3.3.8 Algal Biofuels

Over the few decades, biomasses have been extensively used in the production of biofuels and other bioproducts (Raheem et al. 2018). Depending on the choice of biomass used, the biofuels can be classified into four generations. *First-generation biofuels* are agro based which utilized food crops such as sorghum, sugar beet, maize, sugarcane, soybean, etc., which upon fermentation by yeast produced biofuels like bioethanol and biodiesel (Lü et al. 2011). Utilization of cultivated crops had an adverse negative impact on global food security (Rosenthal 2007). *Second-generation biofuels* utilized nonedible parts of plants such as grass, silver grass, switch grass, *Jatropha*, etc. (Brown and Brown 2013). Algae-based biofuels are *third-generation biofuels* which had no competition with food crops. They also reduced the utilization of land and water and usage of excessive chemicals (Nigam et al. 2010; Raheem et al. 2018). The fourth-generation biofuels concentrate on

Table 3.1 Summary of the various organisms and the biofuel they produce from various substrates

Biofuel	Microorganism	Substrate	Process	Reference
Biobutanol	*E. coli*	Xylose	Elementary mode analysis	Trinh (2012); Peralta-Yahya et al. (2012)
	C. beijerinckii *C. acetobutylicum* *C. sacharoperbutylacetonicum*	Soy molasses, starch-based packing peanuts, gelatinized sago starch	Acetone-butanol-ethanol (ABE) fermentation	Kolesinska et al. (2019); Higashide et al. (2011)
	Bacillus subtilis	Cassava starch	Elementary mode analysis and ABE fermentation	Li et al. (2012); Tran et al. (2009)
Bioethanol	*Lactobacillus sakei LB49*, *Lactobacillus plantarumM24*, *Weissella viridescens LB37*, and *Pediococcus acidilactici M17* *Zymomonas mobilis* *Thermoanaerobacter mathranii*, *T. pseudoethanolicus*, *T. pentosaceus*	Corn, corncob, paper, pine cones using cellulose Hemicellulose	Batch culture method	Soleimani et al. (2017); Yao and Mikkelsen (2010)
Biodiesel	**Microalgae** *Botryococcus braunii*, *Cylindrotheca* sp., *Nitzschia* sp., *Schizochytrium sp.* **Bacterium** *Arthrobacter* sp., *Acinetobacter calcoaceticus*, *Rhodococcus opacus*, *Bacillus alcalophilus* **Yeast** *Candida curvata*, *Cryptococcus albidus*, *Lipomyces starkeyi*, *Rhodotorula glutinis*	Fatty acids from animal fats, vegetable oil, palm oil, wasting oil	Chemical catalysis and enzymatic catalysis using batch culture method	Meng et al. (2009)

(continued)

Table 3.1 (continued)

Biofuel	Microorganism	Substrate	Process	Reference
Biomethane	*Anaerovibrio*, *Aminobacterium*, *Gelria*, *Synergistaceae*, *Longilinea*, *Ruminococcaceae*	Lipids	Anaerobic digestion	He et al. (2018)
Biohydrogen	Microbes from *Eisenia foetida* (lixiviated earthworm) *such as* aerobic bacteria, anaerobic bacteria, nitrifying bacteria, *Pseudomonas* sp., *Bacillus* sp., *Aspergillus* sp., *Actinomycetes* sp.	Agro-industrial wastes such as molasses, bagasses, and vinasses	Anaerobic digestion	Contreras et al. (2018)

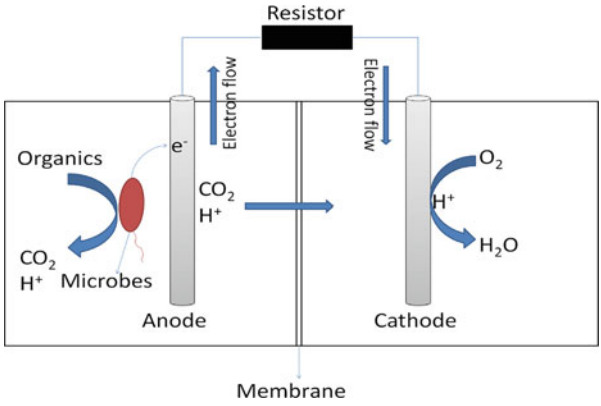

Fig. 3.3 The steps in algal biofuel production from biomass

metabolically engineering and manipulating the biosynthetic pathways of microalgae and bacteria for cost minimization and enhanced biofuel yield (Lü et al. 2011; Dutta et al. 2014; Aziz et al. 2017).

The first step in algal biofuel production is cultivation of algae. Photoautotrophic and heterotrophic modes of algal cultivation are usually preferred. Photoautotrophic method can be done in open ponds/closed photobioreactors. The cultivated algal biomass is then harvested using suitable techniques such as flotation, flocculation, centrifugation, precipitation, sedimentation, and filtration. The lipids from the harvested biomass need to be converted to their relevant biofuels. A variety of conversion techniques are adopted for obtaining biofuels. *Thermochemical conversion technique* involves breakdown of the algal biomass by heat and reformation of the organochemicals to biofuels. *Biochemical conversion* involves fermentation of sugars which results in the formation of biofuels such as biogas, bioethanol, biohydrogen, etc. *Transesterification* of triglycerides in the presence of a suitable alcohol produces biodiesel and glycerol. This step is important in order to reduce the viscosity of the oil produced from algae. The *photosynthetic microbial fuel* cells depend on the oxygen liberated at the cathode which enhances the transfer of electrons at the anode (Saad et al. 2019). Due to the synergistic interaction of fermentation by bacteria at the anode with the oxygen-dependent microalgal photosynthesis at the cathode, appreciable and significant power is generated (Mohan et al. 2014). Various strategies in the production of biofuels from algal biomass are depicted in Fig. 3.3. Most recently a cost-effective *virus-mediated algal cell disruption technique* employing *Paramecium bursaria Chlorella* virus 1 (PBCV-1) has been reported (Zhe and Zhi 2019). Table 3.2 summarizes the various algae, the biofuel they produce from various substrates, and its recovery process.

Table 3.2 Summary of the various algae, the biofuel they produce from various substrates, and its recovery process

Algae	Biofuel	Substrate	Cultivation method	Product recovery method	Reference
Chlorella, *Dunaliella tertiolecta*, *D. salina*, *Haematococcus*, *Chlamydomonas*, *Scenedesmus dimorphus*, *Botryococcus braunii*, *Scenedesmus obliquus*, *Spirogyra* sp., *Chaetoceros muelleri*, *Porphyridium cruentum*, *Euglena gracilis*, *Isochrysis galbana Parke*, *Prymnesium parvum*	Biodiesel	**Heterotrophic cultures:** Utilize glucose **Phototrophic cultures:** Utilize carbon dioxide	Raceway pond and photobioreactor	**Lipid recovery:** Gravimetric methods using organic solvents, Co_2–based solvents, Nile red lipid visualization method, sulfo-phospho-vanillin method, and thin layer chromatography	Mohammady et al. (2015); Chen et al. (2018); Amini et al. (2013); Zullaikh et al. (2019)
Chlorella vulgaris, *Scenedesmus obliquus*, *Chlorococcum humicola*, *Chlorococcum infusionum*, *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Trichoderma reesei*, *Porphyridium cruentum*, *Spirogyra* sp., *Tetraselmis suecica*	Bioethanol	Lignocellulose	Simultaneous hydrolysis and fermentation, simultaneous saccharification and fermentation	**Carbohydrate recovery:** Chemical hydrolysis and enzyme hydrolysis	Lam and Lee (2015); Alvira et al. (2010)
Chlorella vulgaris, *Nannochloropsis oculata*, *Spirulina platensis*,	Bio-oil	Acetate	Hydrothermal liquefaction (HTL)	Lipid recovery methods	Biller et al. (2012); Huang et al. (2016); Alba et al. (2012)

Cyanobacteria sp., *Desmodesmus* sp.					
Anabaena cylindrica, *Mastigocladus laminosus*, *Chlamydomonas reinhardtii*	Biohydrogen	Starch/ glycogen and cellulose	Pneumatic photobioreactors	Dark fermentation	Nayak et al. (2014); Miyamoto et al. (1979); Oncel et al. (2015)
Chlorella vulgaris, *Spirulina* sp., *Arthrospira maxima*, *Euglena gracilis*	Methane	Microalgal biomass	Photobioreactor	Batch digestion and hybrid flow-through reactor followed biomethane potential assays	Buxy et al. (2013); Zamalloa et al. (2012); Inglesby and Fisher (2012); Nguyen et al. (2015)
Euglena gracilis, *Chlorella protothecoides*, *Microcystis aeruginosa*	Oil	Microalgal biomass	Photobioreactor	Batch pyrolysis reactor	Rizzo et al. (2013); Miao et al. (2004)
Nannochloropsis sp., *Chlorella vulgaris*, *Nannochloropsis oculata*, *Nannochloropsis gaditana*, *Spirulina platensis*	Syngas	Carbon	Photobioreactor and raceway pond system	**Thermochemical process:** Gasification, pyrolysis, torrefaction	Onwudili et al. (2013); Khoo et al. (2013); Duman et al. (2014); Sanchez-Silva et al. (2013); Stucki et al. (2009)

3.4 Microbes as Factories for Biofuel Production

The replacement of conventional fuel requires the bulk production of environmentally safe and economically feasible biofuels from renewable energy resources. The usage of microbes with versatile nature to efficiently produce eco-friendly biofuels directly from renewable sources such as biological wastes and biomass can help combat the problem of fuel insufficiency which is a global demand. Recently, research in biofuels has increased tremendously, owing to the metabolic diversity of various microbes in the production of their respective biofuels using various easily available and renewable feedstocks. Carbohydrates are the major source of carbon for producing biofuels. They are obtained from either lignocellulosic biomass or food crops. Other feedstocks for biofuel production may include acetate, lactate, syngas, and glycerol. Additionally, photo-biorefineries which help in conversion of carbon dioxide and light energy into useful chemicals are also being developed (Lindberg et al. 2010; Lan and Liao 2012; Oliver et al. 2013).

Microbes utilize organic substrates and its subsequent metabolism leads to the generation of the most valuable biofuel. However, criteria such as selection of microbes and choice of substrates and the process for biofuel production are of crucial importance. Ethanol production by microorganisms from corn requires utilization of more energy from fossil fuel than to a process where sugarcane is used as the substrate (Goldemberg et al. 2008). Hence, a biofuel that has a better positive net energy balance may be considered as the best suited for commercialization.

Selection of an efficient substrate, for the microorganism to produce biofuels, is definitely a matter of concern. Lignocellulose-containing substrates, namely, plant biomasses and agricultural wastes, can be considered as the most desirable alternatives in comparison to the feedstocks of other types. However, some microbes do not completely degrade lignocellulose into its fermentative constituents, e.g., *Saccharomyces cerevisiae* (Chang et al. 2013).

Lignocellulosic biomasses undergo deconstruction to form biofuel. This conversion starts with a pretreatment which is followed by an enzymatic hydrolysis step or by consolidating these two steps in one reactor (Mosier et al. 2005; Kumar et al. 2009). The process of cellulolytic hyphal penetration can be carried out either by physical or chemical or biological methods or by a combination of all three. The resultant biomass is then hydrolyzed by cellulolytic microbes or by a cocktail of cellulolytic enzymes. (Lynd et al. 2002).

3.5 Metabolic Engineering: A Key Technology for Upscaling Microbial Production of Biofuels

Renewable feedstocks have been extensively researched upon for microbial production of biofuels. Since carbohydrates are the major substrates for biofuel production, they can be obtained either from lignocellulosic biomass sources or from food crops. Other sources of feedstocks for biofuel production are syngas, lactate, glycerol,

acetate, etc. Additionally, recent research for developing photo-biorefineries to convert light and carbon dioxide to useful chemical forms is in progress (Lindberg et al. 2010; Lan and Liao 2012; Oliver et al. 2013). Moreover, initiatives for gas to liquid bioconversion, targeting methane to be used as a cheap resource, have been taken by the Department of Energy (Conrado and Gonzalez 2014). Although various biomass sources and feedstocks have been proposed for biofuel production, cheaper techniques have not been developed yet. The most developed process for biofuel production is ethanol fermentation by using yeast. But owing to its high purification costs and low combustion energy, using ethanol may not be economical. Therefore, scientists and researchers have modified microorganisms to produce new biofuels by altering their metabolic pathways.

Yeast is a widely used organism for the production of bioethanol. But the bioethanol thus produced is considered not to be economically feasible owing to its high purification costs and low energy of combustion. Hence scientists have then begun to metabolically engineer other microorganisms to enable them to produce high-quality advanced biofuels. Some examples include higher alcohols, alkanes from fatty acids, terpene-based biofuels like isopentenols, and fatty acid ethyl esters (Atsumi et al. 2008; Steen et al. 2010; Choi and Lee 2013). Although there are many advancements in the field of biofuel production, commercialization of biofuels still remains a challenge owing to the inefficient productivity of biofuels by the microbes in huge bioreactors and very low profits (Zhang 2009; Lamonica 2014). Therefore, many companies have begun to concentrate more on advanced biofuels which owe high commercial value.

The ATP and NAD(P)H metabolism in the host microbial cell is governed by multiple chemical reactions. To metabolically engineer the microbe to undergo the desired biosynthetic pathway, various enzymatic reactions are required. Current advanced techniques in molecular biology are exploited by biotechnologists to significantly alter the levels of the biosynthetic enzymes to increase the metabolic flux to favor the biofuel synthesis. Various molecular strategies like promoter engineering, directed evolution or modifications of metabolically important enzymes, choosing high copy number plasmids, synthetic scaffolding, optimizing codons, competitive pathway knockdown/knockout, ribosome binding site improvement, genome editing, CRISPRs/TALENs, RNAi approach, etc. allow researchers to metabolically alter the mechanism of biosynthesis in microbes (Dueber et al. 2009; Nowroozi et al. 2014; Sun and Zhao 2013).

In order to improve the metabolic flux toward biofuel production, there are two strategies that are being adopted in metabolic engineering. They are as follows:

1. *Push-pull-block strategy*—where overexpression of certain genes pulls and pushes the metabolic flux toward fatty acid production, while knockout of certain genes blocks the degradation of fatty acids (Atsumi et al. 2010; Kind et al. 2013). In *E. coli* push-pull-block strategy is used for fatty acid metabolism. Overexpression of genes *tesA* and *fadR* is said to pull and push carbon flux to favor fatty acid production. Blocking the degradation of fatty acid is by knockout of *fadE* (He et al. 2014).

2. *Alternate pathways* to modify biosynthesis by reducing the carbon loss in undesirable byproducts. Complete conversion of sugar to acetyl CoA is achieved by designing an oxidative glycolytic cycle and has been developed in *E. coli* (Bogorad et al. 2013).

The strategies used in metabolic engineering mainly focuses on driving the carbon flux toward the desired final product. However, the process is not an easy one. Due to extensive modifications, metabolic burdens are often induced and increased on the host cells which have negative effects like interfering with the cell's growth and the synthesis of the desired final product (Colletti et al. 2011; Poust et al. 2014). Negative effects like incorrect expression of proteins, misfolding of heterologous enzymes resulting in reduction of their activities, heavy metabolic burden on cell growth and productivity due to high copy number plasmids, and waste product production due to metabolic imbalances have been reported (Carrier et al. 1998; Jones et al. 2000; Chang et al. 2007).

Metabolic engineers have started to use synthetic biology tools to alter metabolic flux for biosynthesis at various stages of fermentation. Tools to trigger memory system, toggle switches where two repressors turn off each other, and genetic oscillators are noteworthy to be exploited by metabolic engineers. Precise regulation of gene expression in the presence/absence of chemical and environmental conditions is achieved by these synthetic biology tools (Khalil and Collins 2010). They can also control the host metabolism in accordance to the changes in environment, thereby increasing the host productivity (Zhang et al. 2012). Various metabolic engineering strategies in the development of an ideal strain for enhanced production of the desired product are represented in Fig. 3.4.

Although the current research trend in metabolic engineering focused on improving the carbon flux to the desired final product, certain obstacles need to be addressed:

1. Large amount of ATP is required for the polymerization of protein, DNA, and RNA (Stephanopoulos et al. 1998).
2. Cell maintenance also consumes a large amount of ATP (Hoehler and Jorgensen 2013).
3. ATP and NAD(P)H are also required for the synthesis of biofuel molecules (He et al. 2014; Varman et al. 2014).
4. Alleviation of toxicity in products due to metabolic imbalance (Baez et al. 2011).
5. Underdeveloped large-scale fermentation process for many biofuels.
6. Difficulty in maintenance of optimal growth conditions in large bioreactors.
7. Difficulty in diffusion of gaseous substrates across gas-liquid interface (Blanch and Clark 1997).
8. The microbial cell factories are subjected to metabolic stress and imbalance because of drainage of metabolic precursor and energy (Carneiro et al. 2013).
9. Intensification of stress response, induction of metabolic shifts, and genetic instability have been observed in large bioreactors due to the sub-optimal growth

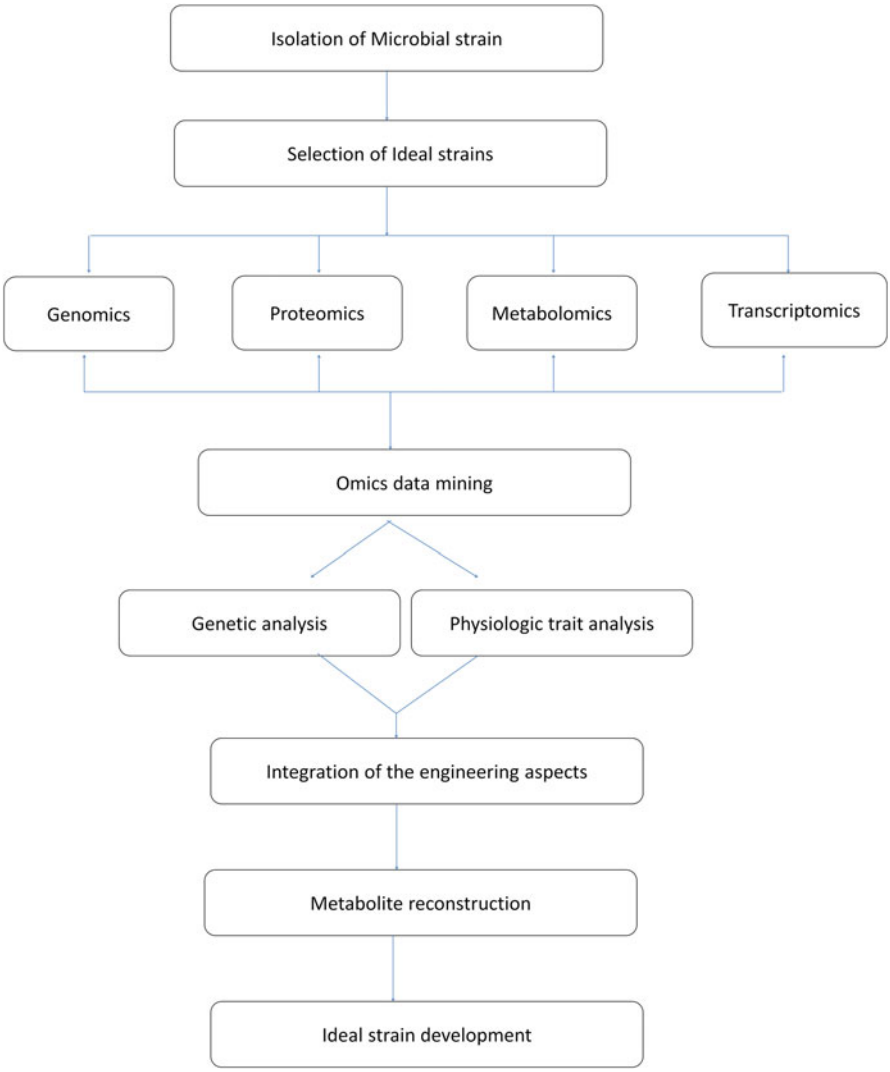

Fig. 3.4 Metabolic engineering strategies in development of an ideal strain for enhanced production of the desired product

conditions. Therefore many of the metabolically engineered "super bug" work inefficiently beyond laboratory conditions.

3.6 Metabolic Engineering: The Future of Microbial Biofuel Production

It is without any doubt that metabolic engineering is a very useful technique to convert microbes into efficient biofuel-producing cell factories. However, identifying the enzyme, targeting the biosynthetic pathway, and altering/replacing the pathway are quite difficult. Identification, assembly, and optimization of novel pathways lead to successfully engineered microbes. The functioning of a certain metabolic pathway is dependent on various factors, and keeping track of the product titers does not help identify problems related to enzyme activities. By exploiting various strategies in metabolic engineering and synthetic biology, the limiting parts of the biosynthetic pathway may be identified (Long et al. 2015; Shabestary and Hudson 2016). Proteomics, transcriptomics, metabolomics, fluxomics, high-throughput sequencing techniques, bioinformatics, and computational biology tools have been widely used as metabolic engineering strategies for enhancement of biofuel production (George et al. 2014; Weber et al. 2015; Wu et al. 2016). Microbial products that are not naturally produced may be synthesized by exploiting the advancements in the fields of metabolic engineering (Chubukov et al. 2016; Koppolu and Vasigala 2016).

3.7 Conclusion

In the current era of scientific advancements, approaches of metabolic engineering are proving to be useful for effective biofuel production using a variety of microbes. In comparison to the conventional strategies for strain development such as mutagenesis, metabolic engineering is accepted to be rapid, precise, rational, and very efficient. Alteration of biosynthetic pathways or an introduction of a completely new pathway can be performed and optimized in microbes to enable to produce the final product of our interest. Optimization of fermentation process for biofuel production may be costly and difficult to achieve in large industrial bioreactors. Therefore, an integration of biofuel fermentation technology and metabolic engineering may be desired for improved metabolism and enhanced heterogeneous gene expression. Additionally, an in-depth understanding of microscopic and macroscopic parameters by the fermentation engineers helps to address the performance gap between the lab-scale studies and its application in industries. However to commercialize the biofuels, the strains used as microbial factories need to be superior to the wild-type strains in their performance. Because of the recent advancements in metabolic engineering, microbes will be able to utilize substrates which they earlier could not. This attribute of the engineered microbes will help improve the economic feasibility of using biofuels as alternative fuels to fossil fuels. Genetic and metabolic engineering in combination with synthetic biology and systems biology are the key to generate highly capable cell factories for production of biofuels.

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Industrial Methanogenesis: Biomethane Production from Organic Wastes for Energy Supplementation

4

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Abstract

There is a limited availability of fossil fuels on earth, which is depleting at a faster pace with growing energy demands worldwide. Moreover, the burning of fossil fuels releases toxic gases which are harmful for all living beings and increase the earth's temperature by global warming. Therefore, there is a need to look for other alternative renewable energy sources which can play a major role in mitigating energy needs. The gaseous biofuel derived from biomass, like methane and hydrogen, is considered to be renewable energy carriers, as there is abundant biomass available on earth. Although the production of methane from biological route is old, there are few considerations which need to be addressed like economical feasibility, feed supply and improvement in production capabilities. The report deals with major issues involved with methane production from

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organic wastes, methanogens and their diversity, production efficiency and sustainability in the near future.

Keywords

Methanogens · Energy crops · Biofuels · Anaerobic digestion · Substrate pretreatment · Hydrolytic enzymes

4.1 Introduction

Globally, fossil fuels are the only source to fulfil the daily energy supply predominantly. The energy needs are increasing continuously with the increase in urban population and per capita income across the globe. However, our fossil fuel reserves are limited and exhausting at a daunting rate which is reflected as decreased production of crude oil in oil-producing countries. So, we are at the verge of energy crisis in the coming future. In addition to this, fossil fuel usage had caused adverse effects on climatic conditions worldwide due to the release of greenhouse gases (GHGs). Thus, there is a thrust in the research towards developing and inventing alternate sources of energy to meet our increasing energy requirements. One of such energy source is "biofuels" obtained from plant-derived feedstocks and crop-generated wastes like sugarcane tops and bagasse, cereal crop straw (wheat straw, rice straw, etc.) and hulls. Presently, one option for biofuel production is some weeds and crops which are termed as "energy crops". They are renewable, eco-friendly sources of energy and have the potential to solve the energy crisis; that is why they are synonymously called "green source of energy". Such energy sources include methane, biohydrogen, bioethanol and biodiesel which can supplement the energy requirement based on the fossil fuels present either individually or in blend form. Gaseous biofuels like methane or biogas can be produced through the method of anaerobic digestion which has its own advantages. The anaerobic route of energy production paves an alternative for waste management through landfilling which is one of hazardous method to get rid of municipal and other waste causing many secondary pollution problems. Additionally, the anaerobic route generates cleaner fuel along with final residue having utility potential as compost in agriculture. Biogas is composed of 60–65% methane, if produced via anaerobic digestion of organic solid waste. The methane fraction is considered as the "real fuel gas" of biogas apart from other hydrocarbons. Such anaerobically derived methane can substitute coal, natural gas and electricity as energy sources at various places including common household usage to industrial production also. However, methane production through anaerobic digestion is affected by several factors. Some of the crucial factors are pH, temperature, substrate and its composition, total solid (TS) and volatile solid (VS) content, inhibitors, toxicants, hydraulic and organic loading rates, etc. Methane production can be quantified using the parameters of

chemical oxygen demands (COD) and biological oxygen demands (BOD) during the anaerobic digestion using the fact that decrement in COD by 1 KG represents 0.35 m^3 methane production at standard temperature and pressure (STP).

In developing countries like India, still landfilling accounts for the main method of solid waste management. Reports predicted annual production of municipal solid waste (MSW) of around 40 million tonnes having composed of biodegradable material utilizable for biogas production through anaerobic digestion. Thus, the present method of tackling MSW in these countries is impractical and requires attention to achieve dual benefit policy of energy production along with MSW proper disposal management. So, owing to the potential of MSW as substrate for energy production, its encouragement had been phrased in many inter-country agreements like the Kyoto agreement. Even presently it has been established theoretically that biogas has potential to tackle 20–30% of natural gas consumption by placing it as energy source via the usage of MSW in anaerobic digestion. Such MSW utilization as previously stated produced nutrient-rich residue beneficial as soil additive and replacing application of chemical fertilizer thus again supporting the economical eco-friendly production. In this report, the process of biomethane production has been discussed along with challenges involved and methane applications.

4.2 Methanogens: Diversity, Morphology and Occurrence

Methanogens are isolated from different habitats including freshwater habitats to hypersaline conditions. Methanogens include a wide variation of organisms having different properties in terms of cultural requirements. Methanogens consist of a large diversity of organisms with diversified habitat in the environments. The genus *Methanohalophilus* consists highly of halophilic methanogens and shows similarities to the aerobic archaebacteria *Halobacterium*. Methanogens could be found at or near 15 °C (*Methanoculleus submarinus*), while others could be isolated from thermophilic conditions, i.e. at 100 °C (*Methanopyrus kandleri*, *Methanococcus jannaschii* and *Methanothermobacter thermoautotrophicus*). Although most methanogens possess almost neutral pH, still some methanogens can bear extreme pH surrounding. Methanogenesis capacity at low pH is nearly about pH = 3.0, but the optimal capacity nearly about pH 6.0. *Methanobacterium* (hydrogenotrophic methanogen) isolated from peat bogs possesses pH as low as 5.0 which assisting the growth of bacteria, while methane produced by bacteria possesses pH below 3.0. There are also some alkaliphilic methanogens, *Methanohalophilus zhilinae*, which have capability to survive in the hypersaline lake (pH 9.2) of Egypt. An interesting fact is that these methanogens are present in the human intestine and rumen of cattle and other ruminants, e.g. methanogens *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* are detected from human faeces using real-time (RT) PCR (Dridi et al. 2009), while *Methanobrevibacter gottschalkii*, *Methanobrevibacter thaueri*, *Methanobrevibacter woesei* and *Methanobrevibacter wolinii* methanogens had been cultured from the

faeces of horse, cow, goose and sheep, respectively (Miller and Lin 2002). Another methanogen *Methanobrevibacter oralis* was isolated in subgingival sites of patients with periodontal disease (Ferra et al.1994) representing there variety of habitats.

Methanogen microorganisms classified to Archaea domain and belonging to phylum Euryarchaeota have interesting trait of methane production under anaerobic conditions. Methanogens have an array of cellular boundary material like pseudomurein, heteropolysaccharide and proteins in place of the peptidoglycan cell wall as found in *Bacteria*. Another methanogen characteristic is the presence of coenzyme F420 having λ_{max} around 420 nm which acts as cofactor crucial for enzyme catalysis of hydrogenase and formate dehydrogenase and allows it to fluoresce blue-green at 470 nm. Coenzyme M or 2-mercaptoethanesulphonic acid is another methanogen coenzyme involved in the process of methane production through methylation, either produced by the methanogens, such as *Methanobacterium*, or acquired from an external source as in case of *Methanobrevibacter ruminantium* (Enzmann et al. 2018).

Methanogens represent a wide variability in cell morphological traits: *Methanobrevibacter ruminantium* are rod-shaped, motile methane producers; *Methanobacterium formicicum* are rod or filament shaped without motility; *Methanomicrobium mobile* is a rod-shaped, motile methanogen; *Methanosarcina barkeri* and *Methanosarcina mazei* are both coccoid shaped and non-motile (Balch et al. 1979). The order Methanosarcinales contains methanogens with cytochromes and has the ability to grow on the broadest range of substrates (Thauer et al. 2008). Additionally, methane production by methanogens uses different substrates, viz. *Methanosarcina barkeri* produce methane from hydrogen, carbon dioxide, acetic acid, methylamines and methanol, whereas *Methanosarcina mazei* utilizes the same substrates except hydrogen and carbon dioxide.

4.3 Methanogenesis: Substrate Characteristics

Solid wastes with rich amount of organic carbon bear good potential to act as source of substrate for methanogenesis. Solid waste includes municipal sludge material, animal manure, food waste, agricultural residues, etc. which can be used for renewable energy generation via anaerobic fermentation.

Utilizing these organic-rich waste and residues as ideal feedstocks and co-digestion of the organic fraction of municipal solid wastes with other co-substrates were reported as an attractive alternative for sustainable energy management. Additionally, the co-digestion concept uses treatment of several wastes in a single treatment vessel having mixture of several waste types which influences the anaerobic digestion positively on one hand and increases treatment efficiency on other hand by increasing methane yield as well as process stability. Methanogenesis by methanogens requires substrate carbon dioxide along with hydrogen as electron donor generally (Enzmann et al. 2018). However, methanogens from the genus *Methanosarcina* grow slowly on hydrogen and carbon dioxide and thus maintain a separate niche by utilizing methanol and methylamines to produce methane

(Patterson and Hespell 1979; Lackner et al. 2018). Likewise, there is a group of formate, which is formed in the production of acetate, used as a substrate for methanogenesis, although it is often converted quickly to hydrogen and carbon dioxide instead. Volatile fatty acids (VFA) are not considered to be involved in methanogenesis as their conversion into carbon dioxide and hydrogen took a long time. In fact, methanogenesis often utilizes the hydrogen and carbon dioxide from carbohydrate fermentation for process, whereas fermentation of carbohydrates results in the production of hydrogen; methanogen microorganisms involved in fermentation allow optimal function and support the complete oxidation of substrates (Pachapur et al. 2019).

4.4 Methanogenesis: Process Details

Methane produced by anaerobic digestion is one of the largely untapped resources. Wetlands, rice paddies, cow intestines, landfill sites and many other places are sites of large amounts of methane that escapes into the atmosphere and contributes to global warming. It is a biological process which catches the eye of many researchers with a hope to utilize methanogenic bacteria for commercial scale. The biological route for methane production is more lucrative, in the economical and ecological aspect. The methanogenic bacteria have potential to degrade any organic waste, agricultural waste and degradable plastic materials converting them into methane naturally. Many industrialized countries around the world spend millions for the proper utilization of their organic waste with less effort (Fig. 4.1).

This process is very advantageous and effective in utilizing energy from the waste products. The recent advancement in the process is continued fermentation with the discovery of effective catalysts which made the process more viable economically.

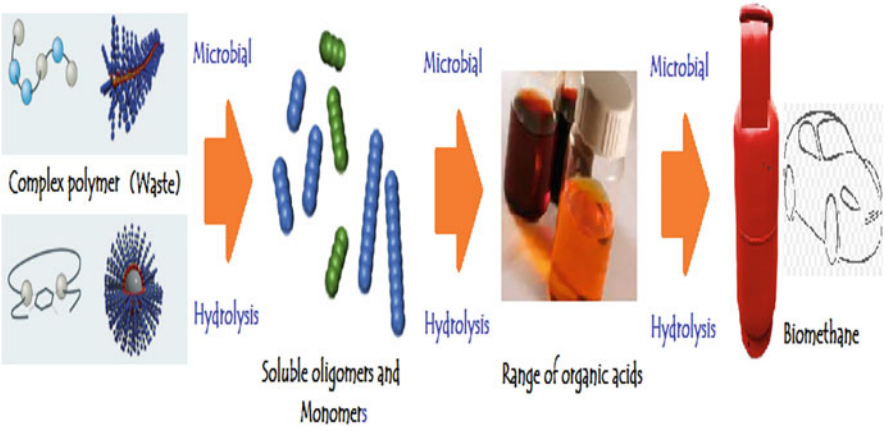

Fig. 4.1 Production of biomethane from organic waste: A general scheme

Anaerobic digestion involves several interdependent sequential reactions along with many other parallel reactions related in complex networks under the absence of oxygen. Also, during the process, the products formed by one group of microorganisms act as the substrates for the next, causing ultimate conversion of organic matter to a mixture of methane and carbon dioxide. Hydrogen and carbon dioxide can be converted to acetate by hydrogen-oxidizing acetogens or methane by carbon dioxide-reducing, hydrogen-oxidizing methanogens. Acetate is also converted to methane by aceticlastic methanogens. Nearly 70% of methane from biogas digesters is derived from acetate. Materials which are converted from microbial biomass accumulate as a residue or sludge which can be used as fertilizer in crop production. Anaerobic fermented digest can be utilized as valuable fertilizer due to the nitrogen content which increased its capacity in short-term fertilization. Methane and carbon dioxide are the principal end products, with minor quantities of nitrogen, hydrogen, ammonia and hydrogen sulphide. Sources that generate biogas are numerous and varied and include landfill sites, wastewater treatment plants and anaerobic digesters. Methanogen processes biogas from a variety of biodegradable waste feedstocks including sewage sludge, municipal waste, food industry wastewaters, agricultural residues and energy crops. These municipal, industrial and agricultural wastes are consumed for energy production which are released every day, in every country, and create serious environmental problems (Sarker et al. 2019).

A complex consortium of microorganisms participates in the hydrolysis and fermentation of organic material. Strict anaerobes such as *Bacteroides*, *Clostridia* and *Bifidobacterium* along with some facultative anaerobes such as *Streptococcus* and *Enterobacteriaceae* take part in the hydrolysis and fermentation. However, the type of substrate available for the process determines the type and extent of the fermentative microorganism involved in the process. In general, mesophilic anaerobic digestion of organic waste is more widely used compared to thermophilic digestion because of the low-energy requirements with high-stability process. About 90% of the full-scale plants, for the anaerobic digestion of biomass, rely on mesophilic continuous one-stage systems (Lin et al. 2011). Another type of digestion operation is preferred in batch where digesters and fresh biomass are filled once and allowed to continue till the completion of all degradation steps sequentially. Many other systems work in two steps; the first is in the continuous digestion and the in second step batch digestion for the digestion of the biomass. There exists a sharp separation between the first phase and second batch system. The acidification proceeds much faster than methanogenesis and a second phase, where acids are transformed into biogas. In terms of handling, this kind of process is easier but has the disadvantage in terms of stringent odours and problem in vacancy at the end of cycle.

4.5 Methanogenesis: Challenges, Solutions and Applications

Biogas production proved its potential as energy source to meet the energy requirements in rural areas, but the expected impact was not made through this technology to support an alternative energy source even though more than two million digesters are established. The major limitations accompanied with the technology are feedstock availability, construction constraints and process failure of anaerobic digestion. However, literature reflects a lot of alternate sources and substrate available to support the biogas production potentially. So, there is a requirement of pilot-scale testing of these feedstocks and developing methods to solve the starting lag in the biomethanation process. These constraints can be solved through the development of substrate-specific biocatalysts and regularity in the supply of high-quality inoculum for the process along with the engineering of realistic practical designs for anaerobic digestion. Thus, there exists a need of coordinated efforts from scientists in different fields to discover a potential solution for the problem in the path of utilizable methanogenesis process and its use for energy production.

There are different approaches, which may be utilized to improve overall efficiency of the process. It includes co-digestion of the substrates, pretreatment of the substrates prior to anaerobic digestion, addition of hydrolytic enzymes, co-production of hydrogen and methane sequentially, development of environment-tolerant microbes for efficient methane production, etc. Some of these approaches are discussed below.

4.5.1 Substrate Co-digestion

Co-digestion involves the utilization of two or more substrates in the anaerobic digestion process which results to efficiency improvement of the digesters. In case of methanogenesis, it had been reported that co-digestion can improve production efficiency by 50–200% which further depends on different types of substrate utilized for the process and operating conditions in digesters (Alvarez et al. 2010). However, success of the technology depends upon the use of the best blending of different substrate which can maximize the production and escape inhibitory processes which ultimately results to profitable methanogenesis. It requires balancing between various parameters of the co-substrate utilized in the process like pH conditions, inhibitors or toxicants generated, micro- and macronutrient contents and biodegradability of organic portion and dry matter content (Deppenmeier 2002). In Europe presently many of the full-scale co-digesters are in operation for the treatment of agricultural and industrial organic wastes for the process of methane production for energy supplementation. In addition to this, it was also found that "digestate" (a semi-liquid residue produced as side product during anaerobic digestion) produced in the method of co-digestion is much rich in nutrients to be utilized as soil additive in agriculture. The reported conditions for efficient anaerobic digestion process are C:N and COD:N ratios of 20 and 70, respectively (Jarvis et al. 2000;

Joblin et al. 2002), along with necessity of alkaline pH to prevent pH decrease during the process which occurs due to the release of volatile fatty acids. In case of nitrogen-rich substrate, lower values of C:N ratio (approx. of 6 to 9) were found to be suitable for the process apart from conditions of ammonia free and total content in the process for the substrate like manure of swine and cattle which were reported to be 1.1 and 4 g N/L, respectively (Kulkarni et al. 2009). Co-digestion of manure with co-substrate like fruit and vegetable waste, MSW, energy crops, sewage sludge and chemicals like glycerine improved methanogenesis supporting the economical efficiency as well as eco-friendly nature of the method (Neves et al. 2006; Nicholson et al. 2007; Parker 2002).

4.5.2 Substrate Pretreatment

Anaerobic digestion requires proper hydrolysis of substrate, and it is the limiting constraint in the process of methanogenesis. Cattle manure which is a common substrate for the process accounts for lower methanogenesis due to its high total solid content, and thus in a typical digester, having typical hydraulic retention time (HRT) in the range of 15–30 days accounts for partial fibre degradation which results to poor yield and increases the sludge retention time (SRT) values for the process (Chang et al. 2011). So, there is need of pretreatment of substrate with different methods of mechanical stress, heating, ozone application, addition alkali, sludge concentration, sonication and sometimes combination of two or more which was reported by researchers time to time Shanmugam and Horan 2009; Whitford et al. 2001; Zhou et al. 2010. These methods disintegrate the cellular assembly of the substrate enabling ease of degradation and digestion in digester enhancing production; however, it also accounts various other constraints in the process like high input of cost, vessel corrosion, foul emissions, etc. which limits pretreatment applicability (Yanagita et al. 2000). Many workers reported about the microwave treatment method which has properties of low-energy consumption, low emission and rapidity in action and thus is presently predicted as efficient method for substrate pretreatment (Zhou et al. 2009).

4.5.3 Hydrolytic Enzymes

For the success of anaerobic digestion, the knowledge of mechanism of hydrolase production at acidogenesis phase is of prime importance as hydrolysis is a limiting factor especially for particulate substrate (Shin et al. 2004). Polymers of substrate are treated by microbial hydrolases during the process in the digester producing their monomeric forms which are utilized by microbes for energy production and cellular synthesis (Fig. 4.2). Several researchers reported about the presence of microbes depending upon the substrate composition utilized in the digester and variation in their population from a variety of substrate used in the methanogenesis process (Rabii et al. 2019).

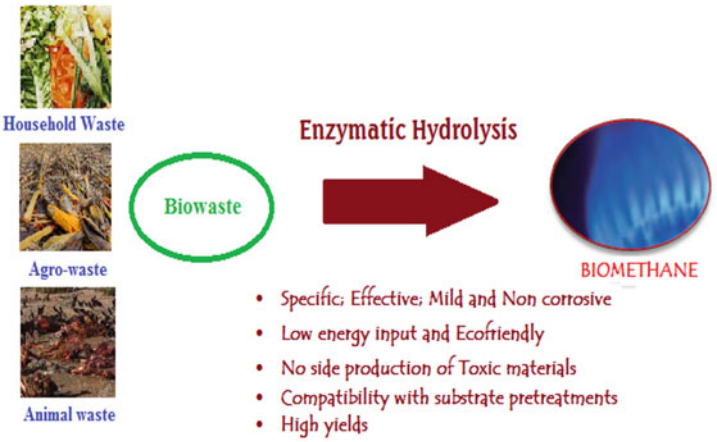

Fig. 4.2 Application of hydrolytic enzymes of microbial sources in methane production and its advantages

4.5.4 Hydrogen and Methane Co-production

In the present scenario, researchers are working on the hydrogen and methane co-production, which is an attractive strategy for energy production. The strategy involves a two-step anaerobic digestion process in the same vessel or digester optimizing H_2 production in the first step and methane production achieved from spent material of the first step. Swine manure along with sludge was reported to be used for production of both hydrogen and methane at an enhanced level, and heat pretreatment of sludge was found to be best followed by alkali and acid treatments (Wang et al. 2009). Similarly, hydrogen and methane co-production was reported from cornstalk substrate using anaerobic fermentation in three stages having additional microbes (*C. paraputrificum*) with an energy recovery of approximately 54% (Lu et al. 2009).

4.5.5 Engineering Eco-Tolerant Microbes

For anaerobic process environ mental factors like pH, temperature, nutrient amount and inhibitor presence are much crucial factors which generally influence the biological processes. The pH of the digester system was affected by co-production of different organic acids during the process. The alteration of pH was resisted by various biological buffers like biocarbonates, proteins and other organic compounds thus escaping methanogens from low-pH conditions. Various strategies are adapted for the pH control, but they have their pros and cons; thus researchers are looking towards the development of acidophilic methanogens which have capability to survive low-pH conditions (Williams and Crawford 1985). Similarly, diversity exploration is required for thermotolerant methanogens as most of them are

thermosensitive and are unable to adapt the altered temperature conditions from optimum (Enzmann et al. 2018). Thermotolerant methanogens are advantageous as it was observed that methane production at higher temperature occurs at twice the rate it was carried with mesophilic ones. However, psychrophilic methanogens and the low-temperature methane production also reported with an advantage of methane production at high altitudes having low-temperature conditions (Wright et al. 2004; 2007) (Garfí et al. 2011).

4.6 Methanogenesis: Optimization Strategies

The table has been compiled (Table 4.1), depicting the recent strategies employed by various researchers to improve the overall biomethane process. It indicates towards achieving enhanced level of methane production by process optimization with a selected substrate. The product obtained by methanogenesis can be put to utilization through various ways like direct combustion and steam generation, in heat and power plants, supplementation of natural gas supply, transportation (for vehicles), etc. Normally, methanogenesis-generated biogas has fuel value of 22,400 kJ/m^3 typically and is put to usage like power generation under combined heat and power (CHP) generation plants. Electric production via CHP is an attractive way, and in the USA alone, 30 projects are involved in electricity generation by the use of methane from waste treatment plants. Biomethane is generally believed to have potential to become an even more widely used fuel, particularly in the automotive industry.

For usage as transport fuel, biogas should have to meet some quality standard norms as it is composed not only of methane but several other impurities which are harmful to presently available vehicle engines. So, there is a need of treatment to get rid of particulate matter and gaseous contaminants like carbon dioxide and hydrogen sulphide which finally raises the methane content up to about 95% by volume. Methods like water scrubbing can be utilized which can remove carbon dioxide (cause of decrease in fuel value) and hydrogen sulphide (causes corrosion), and the purified product can be stored under 200–250 bars for its utilization and supplementation to the natural gas supply owing to its similar properties as of natural gas. Thus, such purified form can be used as natural gas or mixed with natural gas via existing natural gas supply infrastructure and can solve the problem of GHG emission as well as other potent toxicants like benzene a carcinogen to be introduced in the environment (Pohland et al. 2002; Tilche and Galatola 2008).

Table 4.1 Recent process optimization strategies used to improve biomethane production

S. no.	Substrate studied	Process optimization	Process improvement	Reference
1	Mixture of waste activated sludge, fruit waste and cheese whey	Better anaerobic organisms' activity during digestion	Methane production significantly increased by 31% in the reactor	Hallaji et al. 2019
2	Municipal solid waste	Two-stage digester system, i.e. pilot-scale dark fermenter and continuous methanogenic biofilm reactor	72% of total estimated energy recovered in the form of methane gas	Yeshanew et al. 2018
3	Lignocellulosic biomass and poultry faeces	Human urine as buffering agent in place of sodium bicarbonate and water	Five times higher production	Eduok et al. 2018
4	Waste-activated sludge (WAS)	Disintegration of sludge by sonication	Increase in rate of methane production	Zorba and Dilek Sanin 2013
5	Wheat straw	Optimization of a microwave pretreatment	28% compared to an untreated sample	Jackowiak et al. 2011a
6	Cassava residues	Optimization of thermal-dilute sulphuric acid pretreatment by applying response surface methodology (RSM)	Maximum methane yield was 56.96% higher than the control	Zhang et al. 2011a
7	Switchgrass	Mild pre-treatments on the solubilization of lignocellulose	Methane production from SHS was improved by 42%	Frigon et al. 2011
8	Cassava residues	A stable thermophilic microbial consortium with high cellulose degradation ability was successfully constructed and used for pretreatment	96.63% (259.46 mL/g VS) higher than the control (131.95 mL/g VS)	Zhang et al. 2011b
9	Biomass wastes	Hydrothermal pretreatment	The biogas production from pig manure, cow manure, fruit/vegetable waste and municipal sewage sludge increased by 7.8, 13.3, 18.5 and 67.8%, respectively	Qiao et al. 2011

(continued)

Table 4.1 (continued)

S. no.	Substrate studied	Process optimization	Process improvement	Reference
10	Cattle manure	Application of sonication to a mixture of manure	Increased production of biogas by up to 800% (11.6 m3 biogas/tonne) compared to untreated manure (1.4 m^3 biogas/tonne)	Castrillón et al. 2011
11	Switchgrass	Microwave pretreatment	The time required to reach 80% of ultimate volume CH_4 is reduced by 4.5 days	Jackowiak et al. 2011b
12	Municipal solid waste	Enzyme addition	Methane production increased to 200 mL CH4/g VS as compared to 5.7 mL CH_4/g VS for the control	Jayasinghe et al. 2011
13	Energy crops (maize and grass silage) and solid manure	Co-digestion	Increase in the biomethane yield per unit feedstock	Asam et al. 2011
14	Waste-activated sludge	Acid pretreatment	14.3% increase in methane yield compared to untreated	Devlin et al. 2011
15	Kitchen waste	Pretreatment	Increase in methane yield	Ma et al. 2011
16	Rice straw	Phosphate supplementation	Acceleration of the biogasification process by 7–13 days	Lei et al. 2010
17	Digested biofibres	Mechanical, hydrothermal, chemical and enzymatic treatments	Chemical treatment resulted in 66% higher methane production, and combination of steam treatment with NaOH and subsequent enzymatic treatment increased the methane yield by 34%	Bruni et al. 2010
18	Biofibres from digested manure	Steam pretreatment	67% increase in methane production	Bruni et al. 2010
19	Cassava pulp and pig manure	Co-digestion	41% increase in specific methane yield	Panichnumsin et al. 2010
20	Pig manure	Thermo-chemical pretreatments	Increase of 78% biogas and 60% methane production	Rafique et al. 2010

(continued)

Table 4.1 (continued)

S. no.	Substrate studied	Process optimization	Process improvement	Reference
21	Rice straw	Pretreatment with acetic-propionic acid	Methane production can be enhanced by dilute organic acid pretreatment. Moreover, most of the acid in hydrolysates can also be converted into methane	Zhao et al. 2010
22	Wheat straw hydrolysate	Reactor configuration	With continuous stirred tank reactor (CSTR), maximum methane yield of 297 mL CH4/g-COD could be obtained. On the other hand, the same process in up-flow anaerobic sludge bed (UASB) reactor was less efficient	Kaparaju et al. 2009
23	Municipal solid waste and agro-industrial by-products	Co-digested with crude glycerol	The methane production rate increased from 479 mL/d to 1210 mL/d	Fountoulakis and Manios 2009
24	Solid potato waste and sugar beet leaves	Co-digestion	60% higher methane yield	Parawira et al. 2008
25	Activated sludge mixture	Mixtures of enzymes	Increased methane yield (60%)	Davidsson et al. 2007
26	Japanese cedar wood	Pretreated with a white rot fungus, *Ceriporiopsis subvermispora*	Increased methane fermentation of softwood	Amirta et al. 2006
27	Biological sludge	Mixtures of lipase and glycosidic enzymes	Increased methane production (71%)	Wawrzynczyk et al. 2003

4.7 Conclusion and Future Prospects

The future application of biomethane as energy source still requires many constraints to be considered. Especially, developing countries, which import majority of fuel and energy sources from other countries, need to develop ideal digesters for production of biomethane to supplement their energy requirement utilizing agricultural as well as municipal waste for methanogenesis. It is well established that biomethane

has many eco-friendly properties compared to fossil fuels. This biological fuel source has potential to target many economical and environmental problems currently faced worldwide due to fossil fuel consumption. It is not only a cleaner source of energy but also an efficient source of energy to solve the present energy crisis. It starts right from the selection of suitable substrates, which should be cheap, readily available and consistently available round the year. A mixture of substrates could be used which may include municipal solid wastes, biomass feedstocks, kitchen wastes or any other organic wastes. There is also an emerging concept of biphasic production of gaseous biofuel from biomass. Therefore to entirely tap the potential of methane as an energy carrier with commercial viability, a comprehensive approach is required.

Especially, developing economies are feeling a severe pressure because of rising crude importing bills. Therefore, biomethane presently emerges as one of the most important renewable energy sources. The main issues which need to be addressed for efficient and sustainable methane production from organic wastes are biomass conversion to methane production rate, knowledge of the microbial communities in digesters and anaerobic digestion process stability. There is a requirement to develop microbial inocula which could work in a diverse temperature range and make methane production more efficient.

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Recent Trends and Advancements in Biosensor Research for Food Safety

5

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Abstract

A vast majority of food safety concerns are caused by the consumption of contaminated food.

Thus, there is an increasing demand of improved methods for detecting the foodborne pathogens. Traditional microbiological detection and identification methods for foodborne pathogens are well known to be time-consuming and laborious as they are increasingly being perceived as insufficient to meet the demands of rapid food testing. Biosensing technologies have put forward themselves as an alternative for rapid and effective detection of foodborne pathogens. A vast range of signal transducers have been developed in the recent time to detect foodborne pathogens. Their sensitivity and results vary significantly based upon the features of the transducers and the biological materials being used as analytes. However, the development of highly sensitive biosensors for rapid, effective detection and identification of foodborne pathogens for ensuring food safety still remains a challenging task in front of global food safety organizations due to one or other technical obstacles. The present chapter highlights various biosensing technologies available for quick, on-site, and efficient detection of major foodborne pathogens.

Keywords

Biosensor · Obstacles · Transducer · Analyte · Foodborne pathogens · Food safety

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5.1 Introduction

One of the major healthcare concerns for mankind in the modern era is foodborne pathogens causing severe illness and deaths worldwide. There is an increased awareness among people worldwide about food safety. Food safety has become a major health concern globally as foodborne diseases have outspread in both developing and developed countries to alarming levels (Zhao et al. 2014). The increased globalization of food supplies due to cross-country trades has made food safety as the most vital concern (Lan et al. 2017). Unhealthy and unhygienic food can cause various dreadful diseases eventually leading to deaths (Sharma et al. 2015). The current worldwide situation from a health perspective is dire. Currently, the most significant food safety concerns are caused by the consumption of contaminated food. Foodborne pathogens affect food safety at various stages including food manufacturing, handling, food distribution, and finally the consumption by the customers. Thus, monitoring and real-time detection of food contaminants hold utmost importance to ensure the availability of risk-free and contaminant-free foods. The current global situation in the twenty-first century from a food safety perspective is dire. Over the years, foodborne pathogens have become a major factor contributing to society's ill health, thus increasing the morbidity and mortality rate to alarming levels in both developed and developing countries. Foodborne pathogens are basically the microbes such as bacteria, fungi, viruses, and several other parasites that have capabilities to infect humans consuming contaminated foods. Major foodborne pathogens responsible for illness and deaths worldwide include foodborne bacteria such as *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Clostridium perfringens*, *Salmonella enterica*, *Campylobacter jejuni*, *Toxoplasma gondii*, *Listeria monocytogenes*, other Shiga toxin-producing *E. coli* strains (non-O157 STEC), *Norovirus* and *Vibrio* spp., etc. (Velusamy et al. 2010; Zhao et al. 2014). Foodborne illnesses have been exacerbated by these contaminants resulting in a number of dreadful diseases and disorders such as recurring intestinal inflammation, reactive arthritis, blindness, mental disability, chronic kidney diseases, and even deaths. Yasmin et al. 2016 documented that an estimated two million deaths happen every year due to around 200 diseases and disorders like diarrhea, cancer, etc. caused due to consumption of contaminated foods. Thus, the World Health Organization has endorsed food safety as "from farm to plate (and everywhere in between) make food safe" on World Health Day, 2015. The US Centers for Disease Control and Prevention in their reports documented that in the United States, 1 among 6 people get ill per year and approximately 3000 people die because of foodborne diseases (CDC 2011). The US Department of Agriculture in their reports documented an approximate cost of $15.6 billion attributed to illnesses caused due to foodborne pathogens (CDC 2016). Foodborne pathogens are the root cause of severe health issues associated with the consumption of contaminated foods globally. Thus, the effective, accurate, and real-time detection of these contaminants is the only possible measure to ensure global food safety from a public health perspective (Arora et al. 2013). It should be taken care of to maintain proper hygiene and aseptic environment during the production, processing, and packaging of foods

so as to minimize the outbreaks of diseases and disorders as a consequence of consumption of contaminated foods (Arora et al. 2013). Thus, looking at the current global food safety and health concerns, there is a desperate need for developing much efficient methods of foodborne pathogen detection besides improving the existing methods. Biosensing technologies have put forward themselves as an alternative for rapid and effective detection of foodborne pathogens. A vast range of signal transducers have been developed in the recent time to detect foodborne pathogens. Their sensitivity and results vary significantly based upon the features of the transducers and the biological materials being used as analytes. However, the development of highly sensitive biosensors for rapid, effective detection and identification of foodborne pathogens for ensuring food safety still remains a challenging task in front of global food safety organizations due to one or other technical obstacles. The purpose of this chapter is to review the biosensing methods available for quick, on-site, and efficient detection of major foodborne pathogens along with various challenges that need to be addressed as well as improvements that are required to make biosensing devices a real utility in the upcoming future for ensuring global food safety.

5.2 Current Public Health Situational Analysis in Developed and Developing Countries

The current global situation in the twenty-first century from food safety and public health perspective is dire. The world's population explosion and the insatiable quest for advancement by mankind have brought about very grave consequences on man's livelihood. A continuous surge in the world population has led to a huge strain on the available societal food resources. This has been exacerbated by the rapidly increasing populations especially in developing countries. In developing countries, the emergence of slum areas in the periphery of major urban cities has led to an increase in the levels of poverty, poor living conditions, illiteracy, unemployment, crime, violence, alcoholism, substance and drug abuse, prostitution, and smoking, thus contributing to society's ill health. The excessive pervasiveness and recurrence of foodborne diseases worldwide especially in developing countries clearly suggest that food safety concerns need to be addressed. Thus it becomes imperative to ensure quick, effective, and on-site detection of foodborne pathogens so as to minimize their prevalence (Zhao et al. 2014). Thus, in this context, there has been a continuous demand for the development of efficient and quick detection methods for the detection of foodborne pathogens from a global public health perspective. A significant number of microorganisms have been reported with the ability to generate toxins responsible for causing foodborne illnesses. These include microorganisms such as *E. coli* O157, *S. aureus*, *Bacillus cereus*, *Clostridium botulinum*, *C. perfringens*, *Vibrio cholera*, etc. (Fusco et al. 2011). Communicable diseases and mental health issues may rank high among the illnesses. It is not uncommon to confront cases of diarrheal diseases, malaria, respiratory infections such as tuberculosis, HIV/AIDS, psychosis, depression, and suicide. On the extreme end of the

continuum, in the developed countries, are the noncommunicable diseases such as diabetes, cardiovascular diseases, and cancer. These usually come about due to consumption of contaminated foods and contaminated water. The same diseases are also gaining prominence and recognition in developing countries especially among the middle and upper classes.

The current dilemma is to strike a balance between feeding the majority of the increasing population on available food sources and worrying about the possible after effects of consuming the foods if they are having one or the other forms of foodborne pathogens.

5.3 Technologies Available for Detection of Foodborne Pathogens

The extremity and recurrence of foodborne diseases occurring as an after effect of consuming contaminated foods have made it inevitable for the scientific communities worldwide to develop the technologies for quick, efficient, accurate, and on-site detection of major foodborne contaminants even at extremely low levels. Foods contaminated with pathogens have become a major concern worldwide as food safety has a direct interrelationship with economy and society. Some of the available technologies that can be employed for the detection of foodborne pathogens are enzyme-linked immunosorbent assays, microarray-based techniques, and methods based on polymerase chain reactions along with conventional methods of detection. These technologies can be an answer to the food safety issues faced by most of the developing countries which are currently struggling to feed the majority of their population in the wake of limited food availability, changing weather patterns, erratic rains, and land degradation and limitation. However, majority of these methods are time-consuming. The duration of time taken varies from hours to several days to give an output in the form of result. Thus, to overcome these shortcomings, detection based on biosensors plays a pivotal role in quick and effective detection of food contaminants. Biosensing technologies have emerged as an effective and rapid detection method and have extended their utility in a wide range of areas that include food safety, environmental monitoring, and clinical studies. Biosensing devices are reckoned as highly efficient detection methods due to properties like high sensitivity, specificity, quickness, applicability in various fields, and cost-effectiveness (Thakur and Ragavan 2013; Singh et al. 2020). Advances and developments in biosensing technologies have significantly improved the quality of life as they have the abilities to detect even minute levels of analytes under question (Arora et al. 2013). Foodborne pathogen detection using biosensing technologies has attained significant importance in the food sector for ensuring food safety. Biosensors possess the ability to detect low levels of pathogens and toxins making the foods highly contaminated. Biosensing methods possess certain properties like real-time detection, on-site detection, high sensitivity, selectivity, etc. which makes this technology advantageous over conventional methods of foodborne pathogen detection. These salient features give an indication of

biosensing technology being used as stand-alone devices for foodborne pathogen detection in the near future (Zhao et al. 2014).

5.4 Biosensors for Detection of Foodborne Pathogens

Biosensor devices for foodborne pathogen detection in general possess as minimum as three elements which include a biological capture molecule, a measure for converting the interaction between capture molecule and target into a detectable signal, and a readout (output) system (Lazcka et al. 2007; Velusamy et al. 2010). Methods such as enzyme-linked immunosorbent assays and those based on polymerase chain reaction are considered as quick detection methods. However, these methods take several hours to days for reaching at some kind of interpretations and results (Velusamy et al. 2010). The urgency for development of highly sensitive, quick, proficient, and accurate detection methods has paved a way for the development of biosensing technology. Biosensors are basically the sensing devices which can be utilized for analyzing and detecting the substances (analytes) in question by translating a biological response to a detectable signal (Velusamy et al. 2010). Biosensing devices comprise of a biological sensing element coupled to a transducer which translates the biological response to a measurable signal. Biosensors fulfill the requirements of desired features of high sensitivity, rapidness, real-time detection, and economic analysis of analyte under investigation. Biosensor devices are portable, offer on-site detection, and possess the ability for both on-site and in laboratory detection of multiple pathogenic organisms. These characteristics make biosensors highly advantageous over other available technologies of foodborne pathogen detection which otherwise would take several hours for their detection. The abovementioned advantages of biosensing technology have made it possible to ensure correct and on the spot detection of foodborne pathogens present (if any) before consuming the food (Rasooly and Herold 2006). Different biosensors work on different fundaments of analyte detection. Overall sensitivity of a biosensor device relies upon the characteristics of transducer and upon the types of biological materials utilized as biorecognition element for analysis (Palchetti and Mascini 2008). Different biological substances which are used as biorecognition elements in biosensor devices include antibodies, peptides, nucleic acids, bacteriophages, aptamers, etc. Some biosensors utilize labeled probes or reagents for analyte detection (Bhunia et al. 2010). The performance of a biosensor device is evaluated by assessment of parameters like sensitivity, specificity, selectivity, rapidness in detection, size of device, ability to manufacture on large scale, and cost factor (Arugula and Simonian 2014). An ideal biosensor device must possess the ability to analyze undefined samples within seconds and must have the capability of simultaneous detection of multiple analytes. Biosensors have been developed and applied for the detection of various foodborne pathogens (Table 5.1) including *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Bacillus*, *Salmonella*, *Listeria monocytogenes*, and *Cryptosporidium* as well as various microbial toxins such as staphylococcal enterotoxins and mycotoxins (Asiello and Baeumner 2011).

Table 5.1 Foodborne pathogen detection using different biosensors

Biosensor type	Analyte detected	Reference
Optical biosensors	*Salmonella typhimurium*	Seo et al. 1999
Optical biosensors	*Salmonella enterica*	Koubova et al. 2001; Silbert et al. 2006
Optical biosensors	*Escherichia coli* O157:H7	DeMarco and Lim 2002; Waswa et al. 2007
Optical biosensors	Tobacco mosaic virus	Boltovets et al. 2004
Optical biosensors	*Listeria monocytogenes*	Leonard et al. 2004; Hamon et al. 2006; Bierne et al. 2007; Ohk et al. 2010
Optical biosensors	*E. coli*	Su et al. 2005
Optical biosensors	*Cryptosporidium parvum*	Kang et al. 2006
Optical biosensors	*V. cholera*	Jyoung et al. 2006
Electrochemical biosensors	*Bacillus cereus*	Ertl and Mikkelsen 2001
Electrochemical biosensors	*Salmonella typhi*	Rao et al. 2005
Electrochemical biosensors	Rat IgG, HBsAg, HBeAg	Yu et al. 2006
Electrochemical biosensors	*Escherichia coli*	Elsholz et al. 2006
Electrochemical biosensors	*Bacillus anthracis*	Ghindilis et al. 2007; Liu et al. 2008
Electrochemical biosensors	*Bordetella pertussis*	Lodes et al. 2007
Electrochemical biosensors	*Clostridium piliforme*	Goto et al. 2007
Electrochemical biosensors	*E. coli* O157:H7	LaGier et al. 2007; Lin et al. 2008
Electrochemical biosensors	*Bacillus cereus*	Pal et al. 2008; Elsholz et al. 2009
Electrochemical biosensors	*Escherichia coli*	Pohlman et al. 2009
Piezoelectric biosensors	*Escherichia coli* O157:H7	Wu et al. 2007
Piezoelectric biosensors	*Bacillus anthracis*	Bolton et al. 2000
Immunosensors	*Salmonella typhi*	Singh et al. 2005
Immunosensors	*Salmonella* spp.	McEgan et al. 2009
Immunosensors	*E. coli* O157:H7	Li et al. 2012

5.4.1 Optical Biosensors

Optical biosensors present themselves as a sturdy alternate to conventional methods of analysis on the basis of characteristics like high specificity, high sensitivity, relatively small size, and economic feasibility (Luo et al. 2004). Biosensing of analytes using biosensor device basically depends upon an enzymatic system, whereby the enzyme transforms the analyte in question to a product which can be either oxidized or reduced at a working electrode and maintained at a specific potential. An optical biosensor is a compact analytical device containing a biological sensing element coupled to an optical transducer which translates the biological response to a detectable signal (Dongyou 2010). Analyte detection through optical transducers offers advantages like economic feasibility and utilization of electrodes which are biodegradable. Optical biosensing technology can be further classified into various subclasses on the basis of a number of phenomena like fluorescence, phosphorescence, chemiluminescence, refraction, absorption, reflection, dispersion, etc. Over the past few years, several types of optical biosensors have been developed for quick, efficient, accurate, and real-time detection of various foodborne pathogens and toxins in foods before their consumption (Velusamy et al. 2010).

5.4.2 Electrochemical Biosensors

The underlying basic principle in the operation of electrochemical biosensors is concerned with their abilities of detecting distinct molecules. Electrochemical biosensors are specifically utilized for detecting biomolecules like glucose, hybridized DNA, DNA-binding drugs, etc. The electrochemical biosensing technique involves production or suppression of detectable electrons or ions by distinct chemical reaction types (Kovacs 1998). The electrochemical biosensing method of pathogen detection is transduction-based systems which have been utilized for identification and quantification of various foodborne pathogens. These biosensors can be further classified into potentiometric, conductometric, amperometric, and impedimetric types on the basis of the phenomenon in observation such as potential, conductance, current, and impedance, respectively (Velusamy et al. 2010). Electrochemical biosensors can be classified into amperometric, potentiometric, impedimetric, and conductometric responses, based on observed parameters such as current, potential, impedance, and conductance, respectively (Velusamy et al. 2010). Pedrero et al. 2009 reported that electrochemical biosensors principally utilize electrochemical impedance spectroscopy as a method of transduction for simultaneous detection of multiple foodborne pathogens. Biosensors based upon impedance spectroscopy measure the deviations in the electrical attributes of bacterial cells (coupled to or attached to the electrodes) for detecting foodborne pathogens (Yang and Bashir 2008).

5.4.3 Piezoelectric Biosensors

Piezoelectric biosensors utilize immensely sensitive piezoelectric crystals which possess the abilities of detecting even minor deviations in mass. There are two main types of mass-sensitive biosensors: bulk wave devices and surface acoustic wave devices. Piezoelectric crystals start vibrating at a particular frequency upon applying an alternating current with a fixed frequency. This specific frequency at which piezoelectric crystals vibrates depends upon the mass of the crystals along with the fixed electrical frequency. Piezoelectric biosensors have been extensively utilized for pathogen detection, and their performances in studying the affine interactions have been immensely referred.

5.4.4 Immunosensors

Immuno-biosensors basically rely on antibody-antigen-specific interactions. Their work involves detection of antigen binding to antibody by reaction onto the surface of a transducer which in turn translates changes in the surface parameters to a measurable electric signal (Gomez et al. 2010). The real-time measurement of immunological reactions is rather arduous due to the difficulty in the diffusion of antigen onto immobilized antibodies especially in cases of extremely low level of contaminants. However, a majority of immune biosensors generate results within 20–90 min range which is closer to real-time detection as compared to other conventional methods of pathogen detection.

5.5 Future Prospects

The conventional methods of foodborne pathogen detection although have good sensitivity are time-consuming when it comes to practical applicability. This time taken for giving output in the form of results varies from hours to days. The need of the hour is the development of a technology that fulfills the requirements of an ideal pathogen detection method. Thus to overcome these limitation, new technologies are required in terms of efficiency, accuracy, ability to perform on-site detection in real time, and economic feasibility. Biosensor technology is one such method that has overcome the limitations of conventional methods to significant extents. A vast range of signal transducers have been developed for the detection of foodborne pathogens. Even though biosensing technology holds enormous potential for foodborne pathogen detection, several challenges still need to be addressed to make the biosensor technology a real utility in ensuring global food safety. These challenges include factors like being able to perform in the long run, ease in use, cost-effectiveness, access to common people, increasing the sensitivity, increasing the pathogen detection limit, and development of transducers able to detect multiple pathogens in one food sample in real time. The future of biosensing technology will depend upon the applicability and suitability of the biosensors in the long run and the

ability to provide quick and accurate results for on-site and real-time detection and identification of foodborne pathogens so as to meet the consumer demand for safe foods as well as for ensuring global food safety. However, despite the broader applicability and the great potential of biosensing technologies, there is still a great chance for further developments in the near future.

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Bacteriocin: A Potential Biopreservative in Foods

6

Bhuvaneswari Manivel and Subashini Rajkumar

Abstract

Biopreservation method focuses a great attention to food industry and consumers. Antimicrobial peptides were also termed as proteins or polypeptides produced by bacteria of both Gram negative and Gram positive, during their growth and possess antimicrobial activities. Even though bacteriocins are categorized as antibiotics, they are not. Bacteriocins are generally ribosomally synthesized; some are posttranslationally modified. They have a broader spectrum of activity to the closely related strains. Microbes produced bacteriocins during the primary phase of growth, but antibiotics are synthesized only as secondary metabolites. Bacteriocins are generally cationic and low molecular weight, are easily digested by intestinal enzymes and contain a surplus of arginyl and lysyl residue. They are amorphous in nature and showed helical structure when soaked in aqueous solution. Nowadays bacteriocins are widely used in food processing as natural preservatives, and the use of their metabolic products is generally recognized as safe (GRAS). The natural antimicrobial compound undergoes research in a genetic level as alternative to conventional antibiotics which will benefit both the consumer and the producers.

Keywords

Biopreservative · Bacteriocins · Foodborne pathogens · Food spoilage bacteria · Subtilin

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6.1 Introduction

The antimicrobial technique in living framework are utilization of antimicrobial peptides by the intrinsic response of immune of numerous life structures vary from creepy crawlies to plants to people. The evolution of penicillin opens the entry for the utilization of remedial anti-infection agents by the therapeutic networks to follow up on different disease causing microorganisms. In the food processing industry, the utilization of antimicrobial substance to food processed had turned into a conventional weapon in the food conservation methods. Numerous Gram-negative and Gram-positive microscopic organisms synthesize various advanced substances of protein structure (it may be neither proteins nor polypeptides) having an antimicrobial action called bacteriocins. It has increased gigantic consideration as potential biopreservative. Bacteriocins have antitoxin properties; however bacteriocins are regularly not named as antimicrobials (Cleveland et al. 2001). Bacteriocin holds opposing views from most restorative antitoxins in being proteinaceous and having limited specificity against strain of the equivalent or strain of firmly linked species (Schillinger et al. 1996).

To keep up the nature of raw material, various physical and chemical properties of the products were maintained in food preservation to help provide stability and protection. The method of preservation aims to minimize or completely remove the disease cause in food. Numerous trends and safety concepts in food preservations indicate that various types of foodborne illnesses and food intoxication are on the rise today. Nowadays various commercial food products and easy uptake of food lead to high contamination. Currently broad vision and higher understanding capability in numerous microbial interactions raised the usage of biopreservative in the form of enzymes or a proteinaceous compound (Holzapfel et al. 1995).

To enhance the microbial safety in foods, various novel scientific trends were aimed on preservation of food using biological method. The application of microbial metabolic products suppresses or eliminates unwanted microorganism in food. The most prevalent bacteria are none other than lactic acid bacteria (LAB) found in various types of fermented food and generally named as GRAS (generally recognized as safe) microbes. Lactic acid bacteria promisingly remained as the most profitable culture classified as “food grade microorganisms” in different food systems. They are one of the probiotic cultures and offer different medical advantages, safe to man (Aymerich et al. 2000). Currently bacteriocin has extraordinary consideration as a novel food preservative because it is heat-stable and amenable to proteolytic inactivation.

6.2 Ecology of Bacteriocins

Early experimental studies showed that microorganisms have the ability to synthesize at least one or many bacteriocins considered as a specific characteristic feature. The multiplication and survival of a microbe in a specific environment eliminates the competitive pathogen of the same habitat. Few low molecular weight antibiotics or

toxins are hydrogen peroxide, some enzymes which lyse bacteria, organic acids and diacetyl which have the same property as antimicrobial peptides even though it does not function like bacteriocin. The production of bacteriocin by bacteria plays an important role in bacterial population dynamics. On the basis of evolutional and environmental adaptations, various complex interactions take place in a mixed population.

Riley (1998) explained that examination of bacteriocins in natural environments, such as *Lactobacillus plantarum* in green olive fermentations, *Escherichia coli* in guinea pig conjunctivae, and *Streptococcus mutans* in the human oral cavity, has indicated that the competitive advantage is notably increased growth of bacteriocin-producing cells against bacteriocin-sensitive bacteria in the same environments (Riley and Wertz 2002).

The most widely recognized microscopic organism *E. coli* produces colicin. It contrasts from different bacteriocins of Gram-positive microbes in two diverse ways: firstly, development of pore in the cytoplasmic membrane and secondly host cell degradation and suppression of translation mechanism. Nearly 25 different types of colicins were identified from *E. coli*. Among this the majority of *E. coli* cells were highly opposing towards any one colicin, and the remaining population of *E. coli* cells were resistant to all types of colicin synthesized by cells of sensitive nature.

6.3 Bacteriocins

Bacteriocins are antimicrobial peptides which are produced ribosomally as polypeptides. They show strong bactericidal activity, while some are posttransitionally modified. They are produced by huge varieties of bacteria and some archaea and produce a wide variety of antimicrobial compounds. They are a heterogeneous group and use a specific antagonist against pathogenic bacteria. They are usually low molecular weight not often 10 kDa, and they are easily digested by proteases of mammalian gastrointestinal tract, indicating it is safe for human consumption. Commonly bacteriocins are cationic, amphipathic molecules that contain an excess lysyl and arginyl residues (Rodriguez et al. 2003). They are generally unstructured and easily incorporated into aqueous solutions. It forms a helical structure when exposed to solvents such as trifluoroethanol or combined with anionic phospholipid membranes. Likewise, they offer specific advantage in the survival area by eliminating the competitive pathogens in that habitat. Antimicrobials producing bacteria liberate various toxins either chromosomally synthesized or by means of plasmid acquiring the capability of eliminating predominant pathogens.

In recent years many bacteriocins are successfully identified by scientist. They usually inhibit the growth of organism of the same or closely related species. They also inhibit the growth of sensitive cells or kill them by interfering with the synthesis of cell wall or forming pores in the cell membrane (Settanni and Corsetti 2008). Many different microorganisms are known to produce bacteriocins. Bacteriocins are widely studied in gram-positive bacteria (antibiotics, pediocin-like bacteriocins) and

gram-negative bacteria (colicin, microcins). Among them *Lactobacillus* and *Bacillus* species were the well-known producers (Lodewyckx et al. 2002).

Bacteriocins produced by *Bacillus* showed tremendous applications in inhibiting various pathogens when compared with other bacteriocins synthesized by other bacteria. Organisms such as bacteria, fungi or yeast may cause disease to humans as well as animals. When compared to conventional antibiotics, antimicrobial peptides of *Bacillus* showed tremendous advantage in food preservation and showed various environmental applications such as biocontrol of plant pathogens and plant growth promoters.

6.3.1 Antimicrobial Peptides Produced by Gram-Positive Bacteria

On the basis of various ecological as well as evolutionary aspects, the antimicrobial peptides synthesized by Gram-positive bacteria differ from Gram-negative bacteria. Likewise, antimicrobial peptides of Gram-positive bacteria are highly tremendous than Gram-negative (Jack et al. 1995). The inhibitory activities of lactococci towards other lactic acid bacteria are due to special molecule named as a proteinaceous substance or nisin (Heng et al. 2007).

There are numerous kinds of bacteriocin-producing organisms. Among those populations, *Bacillus* and *Lactobacillus* species highly engage themselves in food preservation. It can be discussed below.

6.3.2 Antimicrobial Peptides Produced by Lactic Acid Bacteria (LAB)

One of the major occurring bacteria is lactic acid bacteria. LAB is a Gram positive bacteria, non-aerobic, fastidious, non-respiring cocci or rods, non spore forming; obligate lactic acid fermenter, produce lactic acid as fermentative end product it showed catalase negative, devoid of cytochrome. In the 1900s based on their interaction in various foods, they are a group of bacteria considered to play a vital role as biopreservative. The ideal natural food preservative should have the following criteria: acceptably low toxicity, stable storage and processing time, efficient at low concentration and economically viable (Kindoli et al. 2012). Lactobacilli synthesize preservation factors commonly named as bacteriocin.

Among the numerous types of bacteriocin the discovery of nisin as a food preservative since ancient times. Bacteriocin has been widely used as commercial food preservative and their demand increased in the last century. Many bacteriocins have been isolated and characterized for their antibacterial activity against a wide range of food contaminating bacteria (Sakala et al. 2002).

6.3.3 Bacteriocin of *Bacillus*

Bacillus sp. is ubiquitous, commensal and transient organism in nature. It is one of the most well-known producers, and it is generally recognized as safe (GRAS) organism because it is degraded by proteases in the intestine of human being (Abdel-Mosheim et al. 2010). For this reason, bacteriocins from LAB and bacilli have a great potential as a natural preservative, replacing chemical preservative. The antimicrobial peptides synthesized by microbes possess selective benefits for their producer against competitive microorganism. The use of bacteriocin might be secluded from foods because the producer strain is often pathogenic in nature. Current development in recombinant engineering techniques involves the movement of bacteriocin production genes from the efficient donor of Gram-positive bacteria to Gram-negative bacteria of food grade microorganism.

Bacillus subtilis strains were one of the well-known producers as well as extensive synthesizer of diverse antimicrobial compounds. *B. subtilis* ATCC6633 produces several antibiotics, rhizocticin and lipopeptides, surfactin and mycosubtilin (Sutyak et al. 2008b). The bacteriocin-like substances of *B. subtilis* and LFB 112 exhibit maximum activity against both Gram-positive and Gram-negative bacteria causing a variety of diseases to animals. Bacteriocin-like substances (BLIS) can prevent the growth of many foodborne and spoilage bacteria such as *Staphylococcus aureus*, *E. coli, Salmonella pullorum, Pseudomonas aeruginosa, Clostridium perfringens.*

Bacillus subtilis GB 0365 is known to suppress various fungi such as *Botrytis, Fusarium* sp. and *Pythium* sp. In 1989, *Bacillus licheniformis* synthesize the antimicrobial peptide in an extreme environment while highly suppressing the activity of Gram-positive bacteria. The highest rate of antibacterial activity was observed in bacillocin synthesized by *Bacillus licheniformis* and also against some closely related species such as *B. cereus*. The probiotic *Bacillus clausii* O/C strain secretes proteinase perceptive antimicrobial substance and exhibits strong inhibition against *Staphylococcus, Enterococcus* and *Clostridium* sp.

6.3.4 Mode of Action and Structure of Subtilin

Bacillus subtilis produced a lantibiotic known as subtilin. It is categorized as a type A lantibiotic (Guder et al. 2000), cationic in nature and pentacyclic (Fig. 6.1). Its molecular mass is 3319.56 Da as revealed by the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (Stein 2005). The nisin Z leader sequence shares 57% amino acid identity with the leader sequence of subtilin. Likewise the pro-region of nisin Z has 61% identity with the pro-region of subtilin. Subtilisin has a strong bactericidal activity because it forms pores in the cytoplasmic membrane and cell wall precursors such as lipid II and pyro phosphate peptidoglycan monomers as a docking module (Martirani et al. 2002).

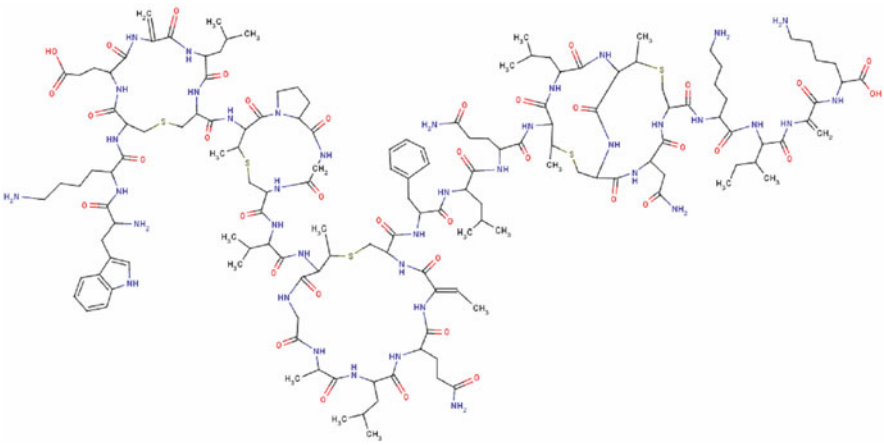

Fig. 6.1 Structure of subtilin

Subtilin molecular formula is –C148H227 N39 O38 S5. This structure is similar to nisin produced by *Lactococcus lactis* (Dodd et al. 1990), likewise, ericins from *B. subtilis* and *epidermin* from *Staphylococcus epidermidis.*

Subtilin is an antimicrobial peptide synthesized by *Bacillus*. The gene which encodes the subtilin harbours (Banerjee and Hansen 1988) nearly a 56-residue peptide precursor that is processed to yield the 32-residue mature peptide. The molecular weight of subtilin is 3319.56 Da as it was revealed by the matrix-assisted laser desorption/ionization time-of-flight MS (Stein 2005). The transformation occurs in precursors by a cyclic dehydration and cross-linking categories to obtain the complete antimicrobial peptide named as subtilin having a remarkable amino acid residues such alanine, methylanthionine, dehydroalanine and dehydrobutyrine. The precursor peptide possesses a leader region having an unusual hydropathic characteristic for an exported protein. Nearly 57% amino acid identity sequence of subtilin also shared by nisin sequence (Beasly and Saris 2004). Likewise, the initial regions of subtilin and nisin possess 61% identity. The structural similarity of subtilin is higher when compared with nisin, their specific operan which encode highly similar proteins.

Subtilin possesses bactericidal activity due to the presence of perforin in cytoplasmic membrane, and cell wall precursor like undecaprenyl pyrophosphate and lipid II act as a hydrophobic carrier module for peptidoglycan monomers as a docking module (Parisot et al. 2008).

6.3.5 Beneficial Role of *Bacillus* sp.

Most member of this *Bacillus* species shows heterogeneity in both phenotypic and genotypic characteristics. It has the ability to destroy a wide range of substrates obtained from both plant and animal sources (Lutz et al. 2006). These organisms are

aerobic, Gram-positive, endospore-forming, rod-shaped bacteria that are characterized metabolically by catalase production. It has the ability to survive in the space for 6 years despite the harsh radiation, vacuum, temperature, etc. It is one of the most important probiotics that existed on the earth for a long time. It performs a vital role in suppressing a systemic pro-inflammatory and autoimmune disorder. It was the first bacterium responsible for determination of cell shape and synthesis of peptidoglycan. It was also identified for localization of peptidoglycan synthesizing enzymes, and it has the capability to synthesize a variety of key nutrients (vitamins, enzymes, carotenoids, lipids, etc.) at absorption site.

It is mainly used for the treatment of gastrointestinal and urinary tract disease. In such a way it functions as an immunostimulatory agent. *B. subtilis* produces carbohydrase (amylase) and protease enzymes. In 1960 this organism was certified by the USFDA (US Food and Drug Administration) due to its non-toxigenic and non-pathogenic nature. *Bacillus subtilis* is also precisely used in a variety of food products. *Bacillus subtilis* acts as a probiotic in everyone's healthy diet. It is highly incorporated in Korean food cheonggukjang. Most of the governmental and non-governmental organizations used dehydrated *Bacillus* culture as feed ingredients or as a silage additive for cattle feed. Spores of *Bacillus* are widely used as a probiotic food supplement.

It is used as a feed for human and feedstock for animals. *B. subtilis* was recognized as QPS (Qualified Presumption of Safety), and the European Food Safety Authority (EFSA 2007) declared it safe for livestock activities due to the lack of surfactant activities and lack of food poisoning toxins such as enterotoxin (Permtoonpatana et al. 2012). It acts as a potential source for the production of various enzymes such as proteases, amylases, some antibiotics, insecticides and also as rennet substitutes.

6.3.6 Categorization of Bacteriocins Produced by *Bacillus*

Like that of LAB bacteriocin, various categorization methods were now available for antimicrobial peptides of ribosomal synthesis. In 1993 Klaenhammer established the categorization of LAB bacteriocins. Similarly Van Belkum and Stiles (2000) and Nes et al.'s (2007) reclassifications were performed. Among different bacterial clades, the bacteriocin produced by *Bacillus* belongs to the lantibiotics, a category of posttranslational modified peptides. On the basis of peptide structure, genetic determinants and biosynthesis mechanisms, lantibiotics are considered as one of the most important antimicrobial peptides (Table 6.1).

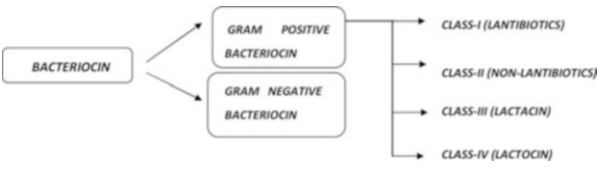

Table 6.1 Comparative features of conventional antibiotics with bacteriocins

Sl. no.	Conventional antibiotics	Bacteriocins
1	Complex ring structure	Proteinaceous in nature
2	Wide spectrum of activity	Narrow spectrum of activity
3	Widely used in clinical applications	More commonly used in medical applications
4	Absence of host cell immune response	Presence of host cell in response
5	Cell membrane is a target site	Cell wall is a target site
6	Specific target	Docking mode of interaction
7	Production cost is too cheap	Production cost is too high

Table 6.2 Classification of bacteriocins of *Bacillus* species and comparison with lactic acid bacteria (Adapted from Abriouel et al. 2011)

Proposed classification of bacteriocins of *Bacillus* species	Examples	LAB bacteriocins (Nes et al. 2007)
Class I. Posttranslationally modified peptides		Class I. Lantibiotics
Subclass I.I. Single-peptide elongated lantibiotics	Subtilin, ericin S, ericin A	
Subclass I.2. Other single-peptide lantibiotics	Sublancin 168, Mersacidin, Paenibacillin	
Subclass I.3. Two-peptide lantibiotics	Haloduracin, Lichenicidin	
Sub class I.4. Other posttranslationally modified peptides	Subtilosin A	
Class II. Non-modified peptides		Class II. Small linear peptides
Subclass II.1. Pediocin-like peptides	Coagulin, SRCAM 37, SRCAM 602, SRCAM 1580	Class II a
Subclass II.2. Thuricin-like peptides	Thurincin H, Thuricin S, thuricin 17, bacthuricin F4, cerein MRX1	
Subclass II.3. Other linear peptides	Cerein 7A, Cerein 7B, lichenin, thuricin 439	
Class III. Large proteins	Megacin A-216 Megacin A-19213	Class III. Large heat-labile bacteriocins

In 2011 Abriouel et al. proposed the classification of *Bacillus* bacteriocins (Table 6.2). Antimicrobial peptides that undergo different kinds of posttranslational modification belong to class I. It can be subdivided into four subclasses. Likewise, small (0.77–10 kDa), ribosomal synthesized, non-modified and linear peptides which are heat and pH stable belong to class II. This class II is subcategorized into three subclasses. The large proteins (430 kDa) with phospholipase activity such as megacins A-216 and A-19213 (Table 6.1) belong to class III.

6.4 *Bacillus* as Biopreservative

The bacteriocin synthesized from the *Bacillus species* suppresses various infections and are caused by most common foodborne pathogens such as *E. coli* and *Salmonella* sp. *Listeria monocytogenes* and *S. aureus* than bacteriocin produced from *Bacillus mycoides* (Sharma and Gautam 2008). The most common Gram-positive bacteria such as *Micrococcus luteus* and *S. aureus* are highly inhibited by two important species *B. subtilis* and *B. pumilus.*

As one of the industrially important species, *Bacillus s*pecies has wide applications in the food industry. The following features depict that the *Bacillus* bacteriocins should be a better alternative to LAB bacteriocin.

In various food fermentation industries, numerous enzymes from *Bacillus* were most commonly used as a starter culture in food fermentation (Ananou et al. 2007). In food processing technology, the bacteriocins produced by *Bacillus* show strong antibacterial activity against various pathogens belonging to either gram-positive or Gram-negative bacteria. It is also effective against fungi. The antimicrobial peptides of *Bacillus* showed wide metabolic diversities such as heat stability, withstand stable pH, use of different organic solvents in food processing. The genetic map of *Bacillus* sp. was well known like *E. coli.* Hence it is very safe for the food industry to synthesize bacteriocin.

6.5 Applications of *Bacillus* Bacteriocins

Antimicrobial peptides offer much advantageous applications in various sectors because of the potential role in food preservation as well as in the medical sector as a therapeutic agent. In recent research, antimicrobial proteins synthesized from *Bacillus* contribute numerous applications in food preservations. It invokes specific interest in research because bacteriocin from Bacilli decreases the limitations of bacteriocin from Lactic Acid Bacteria. It shows wide inhibitory action against various gram-negative bacteria. It even kills fungi. Numerous beneficiaries suggested wide innovations in the field of human health, agriculture, livestock, and food preservation (Abriouel et al. 2011) (Fig. 6.2).

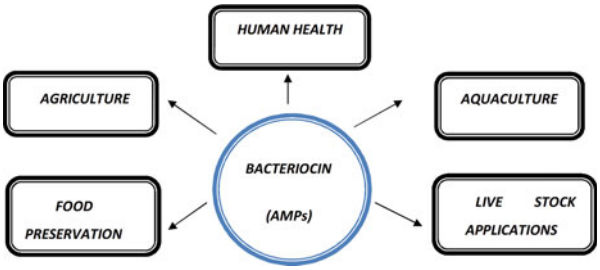

Fig. 6.2 Potential applications of bacteriocin in different sectors

6.5.1 Applications in Human Health

Bacteriocins are considered as a novel source for the control of microbial pathogens and also increase the bacterial resistance to conventional antibiotics (Lawton et al. 2007). Nisin is one of the nontoxic bacteriocins suggested as contraceptive agent. It has a potential to inhibit the growth and colonization of *Helicobacter* which causes peptic ulcer. Similarly, lantibiotic subtilosin shows highest spermicidal activity against spermatozoa from humans exhibiting antimicrobial activity against pathogens such as *Listeria monocytogenes, Gardnerella vaginalis and Streptococcus agalactiae* (Sutyak et al. 2008a). Bacteriocins of *Bacillus* were used to suppress the growth of other bacteria and mean while it offers advantage to microbes in fermenting ecosystem. It does not have the capability to cause vaginal irritation; hence it is more acceptable for human use. The subtilosin A bacteriocin produced by *Bacillus* has proven to be a strong antimicrobial agent against a wide range of pathogens including *Micrococcus luteus, Streptococcus agalactiae* and *Listeria monocytogenes*. It has a huge impact on vaginal pathogens while leaving the healthy microflora to remain intact. Likewise, *B. clausii* as a probiotic strain causes inhibition against *S. aureus, E. faecium* and *C. difficile* (Hill et al. 2009).

6.5.2 Applications in Livestock

Bacteriocin-producing bacilli predominantly act as probiotics in livestock to improve animal health and also inhibit pathogenic bacteria. In 2005 Stern et al. reported that bacteriocin of *Bacillus* sp. has tremendous applications in husbandry. *B. licheniformis* and *B. subtilis* in combination are used to prepare BioPlus 2B. The poultry intestinal pathogens such as *E. coli* and *Yersinia* were strongly suppressed by the spores of *Bacillus amyloliquefaciens*. It is widely used as a probiotic in various animal feeds (Ecobiol, Norel and Nature Nutrition). The non-toxigenic *Bacillus cereus* is used as an animal feed additive because of its probiotic activity. This strain has already been approved by the European Food Safety Authority (EFSA) for animal feed (EFSA 2007).

Antimicrobial proteins commonly used as growth promoters or therapeutic agents and also in animal research are a valuable tool. Nowadays conventional antibiotics were totally altered by bacteriocins in such a way that it reduces the antibiotic-associated problems such as presence of antibiotic residues in the environment and veterinary products and also induced the resistance frequency in bacterial species (Oppegard et al. 2007).

The antimicrobial proteins synthesized by Gram-positive bacteria commonly improved the livestock produced both in vivo and in vitro. Meanwhile, poultry farming, one of the major contaminants as *Salmonella*, was controlled by the use of antimicrobial peptides of *Bacillus*. Microcins produced by *E. coli* hold a promise in reducing the population of Salmonella species in broiler chickens. *Bacillus* species was also commonly used in poultry system as probiotic that showed a maximum decrease of pathogenic bacteria. Current report stated that when colicin

synthesizing the bacteria was inoculated into rumen of cows, it highly reduces the amount of enteric pathogens in the animal. Likewise, lacticin produced *Lactococcus lactis* shows a maximum activity against streptococci and staphylococci in dairy cattle. Current report predicted that probiotic science capable of synthesizing bacteriocin increased the growth rate of swine similar to cattle.

6.5.3 Applications in Food

Currently bacteriocins are extensively used in the food industry especially on food products such as eggs, vegetable and meat. It is highly incorporated as starter cultures. The usage of *Bacillus* bacteriocins specifically focused on targeting food pathogens with special attention. Nowadays many foods are highly incorporated with chemical preservatives (Chen and Hoover 2003). Hence most of the consumers demand for natural foods or minimally processed food, stimulating great interest to antimicrobial agents such as bacteriocin. Dairy products such as milk and soft cheese are specifically preserved by using bacteriocins of *Bacillus*. The bacteriocin cerein 8A synthesized by *Bacillus cereus* 8A was used to control *Listeria monocytogenes* (Bizani et al. 2008). Bacteriocin-like substances synthesized by *Bacillus amyloliquefaciens* strain was used for the biopreservation of poultry milk (Halimi et al. 2010).

Bacteriocins are most resistant to physical factors, while during food processing, they can be neutralized by proteolytic enzymes. It shows higher inhibition to foodborne pathogens. Meanwhile *Bacillus* bacteriocins are considered superior to LAB bacteriocins (Zacharof and Lovitt 2012). It can also be used to enhance sensory properties and improve food quality. Moreover it is integrated into food as sodium acetate or sodium lactate. Apart from these, microbes were predominantly used in the production of various alkaline fermented foods. *B. subtilis* is the only strain which is highly incorporated into East Asian fermented food products (Hosoi and kiuchi 2003). Additionally, certain *Bacillus* species are widely used as inoculums for fermenting soybeans and condiment dawadawa (Terlabie et al. 2006). Likewise, subtilisin was incorporated into fermented soup condiment okpehe. Similarly, *B. subtilis* strain inhibited the growth of various *Bacillus* species (Bhuvaneswari et al. 2016).

6.5.4 Application in Aqua Culture

Marine animal associated microbes were the potent bacteriocin producers. Bacteriocins serve as an eminent tool to reduce the potential pathogens in sea food industry. Antimicrobial proteins totally exclude pathogenic bacteria in water bodies, but enhance the production of inhibitory compounds and also provoke the nutritional state of species by synthesizing the digestive enzymes. The antimicrobial proteins harboured by *Serratia, Pseudomonas, Stenotrophomonas, Photobacterium, Bacillus* and *Aeromonas. Vibrio* species from marine environment was highly screened for

high molecular weight antimicrobial proteins like the compound called hamycin. Various bacteriocins like divercin, divergisin and piscicocin are commonly isolated from *Corynebacterium* (Suzuki et al. 2005).

6.5.5 Applications in Agriculture

Bacilli are commonly found in all sources especially in soil and plants. Majority of bacteriocins inhibit plant pathogens because they possess antibacterial or antifungal activity; hence they are used as biocontrol agents. For example, ericin S produced by *Bacillus subtilis* A1/3 is active against the causative agent of tomato bacterial canker *Clavibacter michiganensis*. From the rhizosphere of a healthy plant, *B. subtilis* 14B was isolated, and it produced Bac 14b, a BLIS active against *A. tumefaciens.* Hence, it is commonly used as a biocontrol agent for suppressing diseases in plants caused by *A. tumefaciens* (Hammami et al. 2009).

Most of the bacilli act as a plant growth promoter and also offer disease resistance in plants. From soya bean root nodules, *B. thuringiensis* strain NEB17 was isolated and used as a co-inoculant with *Bradyrhizobium japonicum* 532C; it enhances nodulations by producing polypeptide. Consequently, the antibacterial peptide thuricin was liberated by *Bacillus*. The introduction of thuricin in soybean caused structural deformities in root hair and also led to curling of root hair tip. The application of thuricin to any part of the plant stimulates the leguminous plant growth in a tremendous manner. The N-terminal amino acid sequence of thuricin was much correlated with other bacteriocins especially from the strain *Bacillus*. The antimicrobial peptides from this organism are thuricin S, thuricin H, bathuricin F4 and cerein MRX1. Meanwhile the other antimicrobial peptides possess the same activity like *Bacillus* and moreover induce the growth of plants.

6.6 Hurdle Technology in Biopreservative

Traditionally, multiple methods of food preservation has developed new ideas to hurdle technology, the presence of microbes in food is not an additive but it also provides some beneficial role. The hurdle technology focused much attention on low-fat content processed food for extending shelf life. The main concepts of hurdles during food safety are water activity, vacuum packaging, temperature, chemical preservative, water activity and UV. In addition to medical applications, bacteriocins are hostile to many important medical and veterinary pathogens. In particular, probiotic microbes liberate bacteriocin in human and animal intestinal tract; hence they protect gut microflora. They have the ability to kill a relatively narrow range of bacteria without causing any harm to natural microbiota, a unique feature when compared to other traditional antibiotics. However, they do not kill many pathogens, but they have the ability to play a very unique role. However, future studies should turn these bacteriocins into practical clinical substitutes to antibiotics and prove their predictable effectiveness, protection and affordability.

6.7 Conclusion

The bacteria from various sources have a capability to produce a variety of bacteriocins. They have a wide range of applications including both in the medical and food industry. To sustain the food quality in a safer manner various bacteriocin synthesizing cultures used as a starter or cocultures in various sectors. The high stability of *Bacillus subtilis* in extreme conditions makes this organism a suitable candidate for probiotic applications either in baked and pasteurized products or in other forms like tablets, capsules and powder. Common *Bacillus* bacteriocins such as subtilin, subtilisin and pediocin were widely applied as preservatives in various dairy products like cheese, kefir, kumis, etc. Further research is required to acquire knowledge in genetic mechanism for bacteriocin production and immunity. Moreover, pharmacological studies and the nature of the molecule after ingestion were required to establish the GRAS status. The use of bacteriocins in combination with natural technology could pave the way to replace the usage of chemical preservatives or could allow less severe processing treatments while still maintaining microbiological safety and quality in foods.

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Utilization of Agro-waste in Pectinase Production and Its Industrial Applications

7

Pitambri Thakur and Gunjan Mukherjee

Abstract

A vast majority of microbial diversity found in nature offers a tremendous scope in the industry. Enzyme, fungal, and bacterial metabolite production is generally done by solid state fermentation. In pectinase production, low-cost agro-wastes are commonly used as substrates that provide a rich source of required minerals and nutrients to living systems. Different agro-waste substrates, apple pomace, citrus peel, tomato peels, papaya peels, cucumber peels, and rice husk, under solid state fermentation are used. The present study relates to an improved process of producing sediment-free, haze-free, and turbidity-free clarified juices, and the main usage of invention will be in the quick enzymatic processing of fruit pulp to a completely clarified juice, which is free from any sedimentation. And other applications such as use of alkaline pectinase in bleaching process in the paper industry will also be the focus of the present study. More emphasis is laid on the utilization of the different agricultural wastes present in India for the production of pectinase for industrial applications and also on the screening and production of strains which are high yielding. Employment of agro-waste and industrial fruit processed byproducts as the substrate, decreases the production cost and recycle the waste by making process cost-effective.

Keywords

Pectinase enzyme · Agro-waste · Fermentation · Microbial pectinase · Pectin

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7.1 Introduction

All around the world specifically in the developing countries, the environmental concern is the same, the problem of discharge of processing operation has gained public awareness. Every year, from agricultural activities, millions of tons of lignocellulosic wastes such as rice husk, rice straw, sugarcane bagasse, and oil are produced, which act as a leading discarding problem. And this lignocellulosic waste has huge amount of pectin, hemicellulose, and cellulose, which are used for the production of pectinase, hemicellulase, and cellulase (Patil and Dayanand 2006). As a researcher if we are thinking to develop any technique, it should be commercially viable and cost-effective. Over the years, enzymes are considered at the center of all industrial processes. And at present due to its industrial applications, they have been the focus of many researchers, to search and produce low-cost enzymes. According to Stutzenberger (1992), pectinases are important industrial enzymes, and with time its market is expanding. Pectinase enzymes are answerable for the breakdown of pectic substances found in plants. Thus, agricultural waste is a good source for pectinase production. Many microbes can produce pectinases, and for the preparation of pectinases, different carbon sources can be used. The history began with considerate knowledge of pectin substance structure and mechanism of pectic substance deterioration. Pectin is present in the middle lamella of plant cell wall and constitutes about 0.5–4% wet weight of fruit and is a plant polysaccharide. Pectinases are mainly alkaline pectinases and acidic pectinases. Various reports have appeared in the course of time on the optimization of microbiological parameters and different fermentation approaches for pectinase production. Addition of chelating agent or pretreatment of plant material enhances the effect of enzymes. Among different pectinases such as animals, plants, fungal and viruses, the most extensive is extracellular pectinases. Bearing in mind the importance of pectinase enzyme, the present investigation is undertaken to find out the use of agro-waste for pectinase production using different agro-waste substrates. Many studies on pectinase production from distinct cheap and low-cost sources such as lemon peel, apple waste, rice husk, wheat bran, and sugarcane bagasse are accessible in the literature (Botella et al. 2007). And as it has high economical condition and great world demand, higher research is needed on cheap and low-cost agro-waste substrates for the production of pectinases. Pectin as carbon source activates pectinase production as it is an empirical process (Hadj-Taieb et al. 2002).

In India sugarcane bagasse, lemon peel, rice husk, wheat bran, and apple are in abundance, so these wastes can be used as most preferable substrates for pectinase production. In this interest, different agricultural wastes used for pectinase production have been disclosed by distinct researchers (Azeri et al. 2010). The earlier studies were limited on cost-effective production of enzymes rather than profitable screening of enzymes. But recent studies have revealed the use of pectin for the screening of enzymes also (Mellon and Cotty 2004; Boccas et al. 1994). During screening of pectinase enzyme, the use of purified pectin is too extravagant for qualitative assessment of enzyme activity. For the screening of microorganisms, these expensive commercial substrates especially pectin have been replaced with

agricultural wastes, i.e., citrus peel and wheat bran. This new approach will be possibly suitable for large-scale screening of microbial aspirations. Thus, the main aim of the present study is to analyze pectinase production from an easily and cheaply accessible agro-waste. Carbon sources specifically of agricultural sources are most suitable source because of their easy and huge availability, cost-effectiveness, and renewability.

7.2 Pectinase Sources and Existence

Pectinases break down pectin present in the cell wall. Activity of pectinase is involved in cell wall metabolic processes such as cell growth, senescence, abscission, fruit ripening, etc. Economical usage of pectinase is in the fruit juice extraction and clarification, and also for the protection and improvement of the quality and hardness of the diverse refined fruits and vegetables. Pectinase is present in plants. In the previous findings, it has been reported in various species such as *Erwinia chrysanthemi* B341, *Saccharomyces cerevisiae*, *Penicillium frequentans*, *Lachnospira pectinoschiza*, *Pseudomonas solanacearum*, *Aspergillus niger*, *Lactobacillus lactis* subsp. *cremoris*, *Lycopersicon esculentum*, *E. chrysanthemi* 3604, *Penicillium occitanis*, *A. japonicus*, *Carica papaya*, *Prunus malus*, *Vitis vinifera*, *Citrus* sp., *Pouteria sapota*, *Rhodotorula* sp., *Malpighia glabra* L., and others (Semenova et al. 2003).

7.3 Pectinase and Its Substrate

Pectinases are the enzymes answerable for the breakdown of pectin found in plants and based on their ability for substrate utilization, divided into pectin and pectic acid different groups (Table 7.1). Pectin acts as the substrate for the pectinase enzymes. Pectin is present in the middle lamella of plant cell wall and constitutes about 0.5–4% wet weight of fruit (Table 7.2). Fungi, bacteria, plants, and yeasts can be different sources of pectinase enzyme. Pectinases' industrial applications were developed because microbial enzyme production is cost-effective. Among these enzymes, endopectinase has special emphasis acting on pectin. Endopectinase breaks down pectin into small molecules, thereby reducing viscosity and filtration time and increasing fruit juice extraction efficiency (Jayani et al. 2005: Gunmadi and Punda 2003).

7.3.1 Substrates for Pectinases

Solid substrate of enzyme can be used as it gives excellent boost to the cells which are growing. For the microorganisms' growth, all required nutrients shall be implemented. Such factors as moisture levels and particle size should be given attention. The cell wall of a plant has complex macromolecular structure that

Table 7.1 Various agro-waste sources of pectinase and its different types of fermentation conditions

S. no.	Different types of agro-waste	Types of fermentation conditions	Enzyme yield	References
1	Citrus waste	pH 8 Incubation time 24 hours	240 μM/ML	Mohandas et al. 2018
		pH 7.5 Incubation time 72 hours Temperature 37 °C Submerged fermentation	0.67 U/mL	Roy et al. 2018
		Temperature 27 °C	221 U/mL	Zhang et al. 2018
2	Wheat bran	pH 5.0 Temperature 30 °C Submerged fermentation	90 μ/gds	Amin et al. 2017a
		pH 3.0 Temperature 35 °C Submerged fermentation	110 μ/gds	Amin et al. 2017b
		pH 7.5 Incubation time 48 hours Temperature 50 °C Solid state fermentation	210.3 U/g	Oumer and Dawit 2017
3	Rice bran	Temperature 33 °C Incubation time 129 h Solid state fermentation	565 U/g	Wong et al. 2017
4	Orange peel	Temperature 30 °C Incubation time 7 days Solid state fermentation	11,063 μ/mL	Handa et al. 2016
		pH 5.5 Temperature 30 °C Incubation time 5 days Solid state fermentation	117.1 mM/ml/min	Ahmed et al. 2016
		pH 5.5 Incubation period 4 days Temperature 30 °C Solid state fermentation	325 μ/mL	Irshad et al. 2014

safeguards and protects cells from harsh environmental conditions and also accommodates different stages of development (Caffall and Mohnen 2009). There are three major components of a cell wall of young plant: cellulose, hemicellulose, and pectin. The microfibrils of cellulose strengthen the cell wall, whereas hemicellulose along with pectin helps in cementing the cellulose network. Cell growth, differentiation, maintenance of rigidity, and integrity of plant tissues are also the basic tasks of pectin (Horikoshi 1999). The utilization of peels and rinds from

Table 7.2 Percentage of pectin content in fresh fruits and in dried parts of plants

Material	Percentage of pectin as calcium pectate	
	Fresh fruits	Dried parts of the plants
Apples	0.5–1.6	–
Apricots	0.7–1.3	–
Bananas	0.7–1.2	–
Citrus peel	–	30–35
Currants	0.9–1.5	–
Guavas	0.7–1.5	–
Grapes	0.2–1.0	–
Lemon peel	–	35.5
Peas	0.5–0.8	–
Peaches	0.3–1.2	–
Pineapple	0.3–0.6	–
Potatoes	–	2.5
Strawberry	0.6–0.7	–
Sugar beet	–	20–30
Tomatoes	0.2–0.5	–

agroindustrial waste for pectinase production lessens the environmental pollution and also adds up to the economical process. Watermelon (*Citrullus lanatus*) as a commercial, tropical fruit in the world is now being used to extract juice which generates waste. The waste is an efficient source of production of enzymes like pectinase.

7.3.2 Pectin: Structure and Distribution

Pectin is present in the middle lamella of plant cell wall and constitutes about 0.5–4% wet weight of fruit and is a plant polysaccharide. And yet its functions are not fully inferred (Willats et al. 2000). Pectin polysaccharides are important cell wall components, and their industrially significant extracts are for pharmaceutical and the other commercial purposes. Pectin is used as a jellifying agent for the processed food products. And in acidic milk drinks, pectin is used as a stabilizer. And according to the source species, location, developmental stages, conditions of extraction, environmental state, and the structure of pectin differ. The detailed analysis of pectin is a difficult task because of these structural complexities and variations. However, the exact architecture of the single element of pectin extracts has been characterized. The basic structure of pectin contains (1–4)-β-D-GalA (galacturonic acid), homogalacturonan (HG), (1–3)-b-D-xylose-substituted xylogalacturonan (XGA), and rhamnogalacturonan II (RG-II). The first-line architecture of pectin is very complicated as it contains many new uncommon sugars and till now is not fully characterized. Based on plant species, the degree and allotment of esterification vary. And this is helpful in controlling the extent of the gelation, which is caused by divalent cations. Pectins consist of rhamnose which is present in

rhamnogalacturonan II. But still within the cell wall, pectic substance arrangement is not clear (Wilson et al. 2000).

7.3.3 Pectic Corpuses

Pectin substances are made up of pectin and pectic acids. Demethylated pectin is noted as polygalacturonic acid. At the C-2 or C-3 position of the main chain, pectin substances are formless; about 200–400 constituents are found with a degree of polymerization. Substituents can be sugar such as D-galactose, L-arabinose, L-mannose, and D-xylose, or non-sugar (acetyl). According to Sakai et al. (1993) on the basis of pectin source, branching type is different in pectin and at the early growth phases in young cell walls the formation of pectin take place in the Golgi apparatus from UDP-D-galacturonic acid. In comparison to lignified tissues, pectin is present in young actively growing tissues at high percentage. The amount of the pectin is less than 1% in higher plants. According to Horikoshi (1990), ripe pears' cell walls contain 11.5% pectic residues, 3.5% galactan, 16.1% lignin, 21.4% glucosan, 1.1% mannan, 21% xylan, and 10% arabinan.

7.3.4 Biosynthesis of Pectin and Pectic Substances

The initial steps of polysaccharide synthesis take place in the endoplasmic reticulum and next in Golgi vesicles, and after that assemblage of the molecules takes place in the cell wall. Few pectic biosynthetic enzymes were located in the Golgi apparatus. Due to complexity of pectin structures, large numbers of enzymes are required for the synthesis of these polysaccharides. Mohnen in 2008 have concluded that 67 distinct methyltransferases, glycosyltransferases, and acetyltransferases are required for the biosynthesis of pectin, with acceptable beliefs about the substrate specificity toward biosynthetic enzymes.

7.3.5 Sources of Pectin

Traditional and commercial sources of pectin are citrus peel and apple pomace. Citrus peel is the most adopted material for pectin production because it has high pectin content and shows good color properties. Citrus peel must be unlimited. Lime therapy of the peel hydrolyzes all the pectin to pectic acid.

7.4 Microbial Pectinase Sources

Pectolysis is the most significant process for the plants, as it shows vital act in cell elongation and fruit ripening. According to Lang et al. (2000), pectolysis is more significant in plant pathogenesis and symbiosis. Very common sources of microbial

pectinases are bacteria, yeast, and fungi, particularly *Aspergillus* species (Table 7.3). By removing pectin residues, pectinesterase is able to deesterify pectin. Pectin depolymerases easily split the main chain into polygalacturonase (PG) and pectin lyase (PL). Pectinases account for 45% of total enzyme handling. Pectinases are phytopathogenic substances (Yadav et al. 2005). Pectinases derived from microorganisms are more in use due to cheap production, easy gene manipulations, and faster product recovery and are usually free from harmful substances as compared to plant- and animal-derived pectinases.

7.5 Classification of Pectic Enzymes

On the basis of action of pectinase on the substrate, pectinases are further classified into three main categories such as polygalacturonase (PG), pectinesterase (PE), and pectin lyase (PL). Complete classification of various pectic enzymes is given in Fig. 7.1. And on the basis of optimum pH required for the enzyme activity, these are classified into two categories: acidic pectinases and alkaline pectinases. Liquefaction and fruit juice clarification are main applications of acidic pectinases (Kaur et al. 2004).

Microbial production of pectinases is largely considered (Favela-Torres et al. 2006). According to Singh et al. (2011), the major source of microbial pectinase is fungus especially *Aspergillus* species. At present, analysis of new microbial isolates has been the central focus of researchers due to its cheap production cost (Phutela et al. 2005). And due to pectinase potential application in the industry, its demand with high stability and new characteristics is increasing commercially. Thus, literature says that pectinase enzyme is isolated from agro-waste or spoiled fruit sources and different locations.

Table 7.3 Microbial sources of pectinase

S. no.	Microbes	References
1	Bacteria (a) *Xanthomonas malvacearum* (b) *Bacillus licheniformis* (c) *Bacillus subtilis*	Papdiwal and Deshpande 2000 Kapoor et al. 2000 Ward and Forgarty 2010
2	Fungi (a) *Aspergillus niger* (b) *Aspergillus japonicus* (c) *Aspergillus fumigatus* (d) *Penicillium viridicatum* (e) *Rhizomucor pusillus*	Dinu and Dan 1994 Semenova et al. 2003 Phutela et al. 2005 Silva et al. 2002 Henriksson et al. 1999
3	Yeast (a) *Saccharomyces cerevisiae* (b) *Wickerhamomyces anomalus*	Omojasola and Jilani 2008 Martos et al. 2013
4	Actinomycetes	Bruhlman et al. 1994

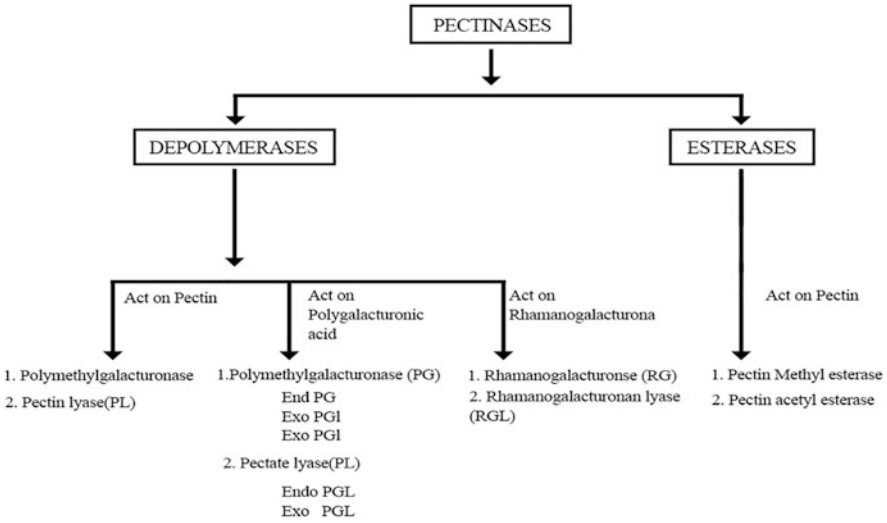

Fig. 7.1 Pectinase classification on the basis of mechanism of action on various pectin substrates

7.6 Applications of Pectic Enzyme

According to the availability of physical conditions, application of pectinolytic enzyme varies. For many years, pectinases are used in several traditional processes such as oil extraction, industrial wastewater treatment containing pertinacious material, textile and plant fiber treatment, and tea and coffee fermentation. Kertesz in 1930 detected the first commercial application of pectinases. Pectinases have enriched applications in fruit juice processing industry as quality enhancers (Junwei et al. 2000). Pectinase has a significant role in clarification and extraction of fruit juices according to Kobayashi et al. (2001), and its use in fruit processing and agricultural wastes has become noticeable. This enzyme is also significant to increase yield and for the removal of fruit peels (Sanchez and Demain 2002). Figure 7.2 represents different applications of the pectic enzyme. According to many present reports, pectinases have instant application in liquefaction, extraction, cloud stabilization, maceration, gelation, and clarification processes (Kareem and Adebowale 2007; Rajagopalan and Krishnan 2008). And one application of pectinolytic enzymes was observed in the case of production of red wine; before addition of wine yeast, pectinolytic enzymes were added, and a high-quality wine with respect to color and turbidity was obtained. And the pectinase processed wine has better stability as compared to the traditional one. The following are the different industrial applications of pectinases.

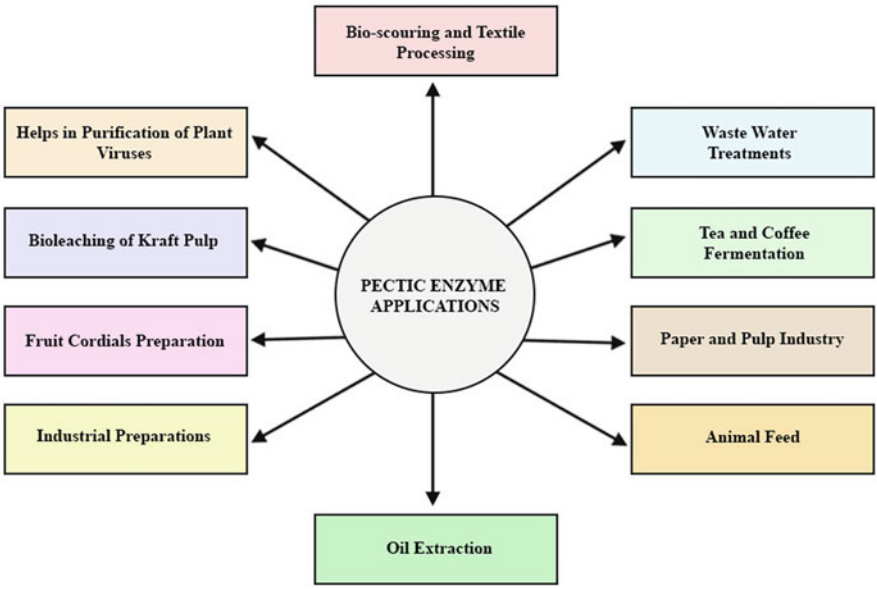

Fig. 7.2 Applications of various pectic enzymes in different industrial and biotechnological divisions

7.6.1 Bio-scouring and Textile Processing

At present use of specific enzymes for different textile processing applications is the thrust because of their ability to replace hard chemicals (Dhiman et al. 2008). Scouring is the process of removing non-cellulosic substances present on the surface of the plant-derived fabrics specially cotton. Bio-scouring is an innovative process in which specific enzymes are used for the eradication of contamination from the fiber without interfering cellulose degradation. Nowadays, cellulase and pectinase enzymes are used for the scouring process, earlier same process was carried by harsh chemicals, due to the employment of enzymes this process is known as bio-scouring. Pectinase breaks the cotton cuticle by digesting the pectin and removing connection between the cuticle and the body of cotton fibers. Due to increasing environmental regulations and difficulties, textile industries are compelled to find eco-friendly processes. In alliance with cellulase, amylase, lipase, and hemicellulase, pectinase is used to replace caustic soda for the removal of sizing agents from cotton in a secure and environment favorable way.

7.6.2 Plant Bast Fiber Degumming

According to Chesson (1980), for the degumming of flax, ramie, jute, and coconut coir, pectinolytic enzymes are extensively used. And for this process mainly alkaline

pectinase is used. Industrially, two basic forms of retting are used. Dew retting is an aerobic mechanism for 2 to10 weeks; plant fodder is dispersed on the ground and uncovered to the fungal and aerobic bacterial action. From dew-retted plants, many species such as *Aspergillus, Cladosporium, Penicillium,* and *Rhodotorula* have been isolated and neutrophilic microorganisms are mostly used for degumming (Bruhlman et al. 1994). These strains can cause heterogenous contaminations at industrial level, so many experiments were conducted by Cao et al. (1992), and described the *Bacillus* sp. (strain NT-33) isolation at pH 10.0, with its importance in the activity of degumming enzymes and also act as an advantage for the prevention of contamination for the development of an open fermentation process. *Bacillus* sp. (strain NT-33) after 24 h of fermentation and under alkaline conditions proved to remove more than 70% of ramie gun. Similarly, from actinomycetes, pectinolytic enzymes have also shown good interaction and results for the degumming effects, which lead to fine separation of bast fiber. As the quality of degummed fibers was far better as per standards, the textile industry was satisfied by this technique.

7.6.3 Wastewater Treatments

Wastewater produced from many industries contains pectinaceous substances that are hardly disintegrated by microorganisms (Tanabe et al. 1986). Pretreatment of wastewater with pectinolytic enzymes facilitates removal of pectinaceous materials and renders it for disintegration by activated sludge treatment. According to Tanabe and Kobayashi (1987) for the pretreatment of the wastewater containing pectinaceous substances, one species, that is, *Erwinia carotovora* FERM P-7576, is considered to be appropriate.

7.6.4 Tea and Coffee Fermentation

For tea synthesis fungal pectinases are mostly used. Enzyme treatment stimulates tea fermentation, and to evade the tea leaf damage the enzyme dose should be fixed. Coffee fermentation is performed to expel the mucilage covering of the coffee beans using pectinolytic microorganisms (Soresen et al. 2000). These beans are then washed, filtered, and sprayed with the fermentation liquid.

7.6.5 Paper and Pulp Industry

In the present papermaking process, retention aids are acquired to accelerate the water drainage. Pulp and paper mills have started using enzymes in their manufacturing processes. *Bacillus* sp. and *Erwinia carotovora* produce alkaline pectinases (Palomaki and Saarilahti 1997), and it is used for *mitsumata* bast due to its strong macerating property (Tanabe and Kobayashi 1987). Japanese paper preparation is done with these retted basts (Horikoshi 1990). All the while in the

papermaking process, pectinase depolymerizes pectins and afterward lowers the cationic need of pectin solutions and the filtrate from peroxide bleaching. According to Holbom et al. (1991) burdensome interfering polysaccharides are solubilized by alkaline peroxide bleaching of pulps. Galacturonic acid polymers can be depolymerized by pectinase (Reid and Ricard 2000).

7.6.6 Animal Feed

Pectinases are used for the production of animal feeds as enzyme cocktail. By breakdown of nonbiodegradable fibers, pectinases liberate blocked nutrients and, by increasing absorption of nutrients, reduce the feed viscosity.

7.6.7 Oil Extractions

Different kinds of oils such as palm, rapeseed, sunflower, kernel, coconut, and olive are extracted by conventional method using hexane as an organic solvent which is a possible carcinogen. In recent times, according to West (1996) in the olive oil preparation, the cell-wall-hydrolyzing enzyme preparation has been started for use. Thereby enzymes are added to release oil at the time of olive grinding process.

7.6.8 Industrial Preparation of Microbial Pectinase

For varied industrial applications, certain microbial pectinases have been commercialized. At industrial scale *A. niger* and *B. subtilis* are the large-scale sources of pectinase enzymes for Nordisk (PectinexTM, Pectinex SP-L) (Perrone et al. 2006; Maiorano et al. 1995). Due to the ample industrial application of pectinases, different restoration mechanisms have made microbial production of enzymes cheap (Gummadi and Panda 2003). Therefore, solid state fermentation (SSF) was tried using different carbon sources such as banana peel, orange bagasse, wheat bran, and sugarcane bagasse separately. Production of the industrially essential pectinase enzyme was tried in distinct combinations to find out suitable and cost-effective innate source for pectinase preparation. Use of low-cost agricultural waste not only commercializes the product but also recycles waste and reduces the capital investment. A multistep process such as soil sample screening from agro-wastes, bacteria, organized fermentation processes, employment of techniques like strain improvement to advance the yield in an integrated way for the fruitful pectinase production is required.

7.6.9 Fruit Cordial Preparation

Pectinase enzyme plays a vital role in the orange juice cordial production and hastens lime juice process (Alkorta et al. 1998). Use of pectinase reduces the processing time from 4–6 months to 4–6 days and helps in eliminating the cost of storage of juice. By adding pectinase enzyme to lemon juice at 1% level at 25–35 °C for 48 h, a sparkling clear product is obtained.

7.6.10 Agricultural Substrate Saccharification Process

Complex molecules of the agroindustrial waste are bioprocessed into simple sugars and finally converted into fermentable sugar substrates or bioethanol. *Landoltia punctate* (duckweed) when treated with pectinase dose of 26.54 pectin transeliminase unit/g mesh at 45 °C for 5 h increased 142% of glucose content in comparison with untreated mesh. And this glucose was used in ethanol production at concentration of 30 ± 0.8 g/L by using duckweed as feedstock. Pectin-rich agroindustrial wastes are also hydrolyzed by pectinases in biorefineries.

7.6.11 Bioleaching of Kraft Pulp

According to Kirk and Jefferies (1996), pectinases have increased demand in paper and pulp industries with advancements in biotechnology. Yellowing of paper takes place due to presence of pectins which leads to its decreased dewatering features. With biotechnology advancement, the use of enzymes for biobleaching and paper-making has increased in many countries (Reid and Ricard 2000; Bajpai 1999). The enzyme pectinase depolymerizes polygalacturonic acids and lowers demand of cations and the filtrate from peroxide bleaching of thermos-mechanical pulp. The enzymatic bioleaching process results in production of reduced organochlorine compounds in the effluent and even attains the same level of brightness of pulp as obtained by conventional methods.

7.6.12 Helps in Purification of Plant Viruses

Pectinases have an ample role in the regulation of disease-causing pathogenic microbes (Ten Have et al. 2002). Different species such as *Aspergillus flavus, B. cinerea*, and *Claviceps purpurea* have shown positive effects. Normally, virulence causing different extracellular enzymes may be secreted by fungal pathogens (Wanyoike et al. 2002). The clear-cut role of many extracellular enzymes is still questionable. But the interruption in the genes encoding enzymes for the respective fungi resulted in the reduction of virulence (Voigt et al. 2005). In many reports there was detection of interrelationship between the pectic enzymes, disease symptom, and relative virulence.

7.7 Agro-waste for Pectinase Production

Every year, millions of tons of lignocellulosic wastes such as rice husk, rice straw, sugarcane bagasse, and oil empty fruit bunch are generated from agricultural, agroindustrial, and forestry industries, which pose a major disposal problem. These lignocellulosic wastes have a huge amount of hemicellulose, cellulose, and pectin, which are used for the production of hemicellulase, cellulase, and pectinase, respectively (Patil and Dayanand 2006). According to Stutzenberger (1992), pectinases are important business enzymes and account for 10% of universal commercial enzymes, and their retail is expanding with time. Pectinases are a group of enzymes answerable for the breakdown of pectic substances found in plants. Thus, agricultural waste is a good source for the generation of the pectinase. Many microorganisms such as bacteria, yeast, and fungi can produce pectinases; different carbon sources can be used for the production of pectinases. Many studies on the pectinase production from distinct cheap and low-cost sources such as wheat bran, lemon peel, apple waste, rice husk, and sugarcane bagasse are accessible in the literature. In India sugarcane bagasse, rice husk, wheat bran, apple peel, and lemon peel are in abundance, so these wastes can be used as the most preferable substrates for pectinase production. In this interest, different agricultural wastes used for the pectinase production have been disclosed by distinct researchers (Azeri et al. 2010). Previous studies were limited on cheap production of enzyme in place of profitable screening of enzymes. But recent studies have revealed the use of pectin for the screening of enzymes also (Mellon and Cotty 2004; Janani et al. 2011). During screening of pectinase enzyme, use of the purified pectin will be too extravagant for partial assessment of enzyme activity. For the screening of microorganisms, these expensive commercial substrates especially pectin have been replaced with agricultural wastes, i.e., citrus peel and wheat bran. This advanced approach may be possibly suitable in the screening of large-scale microbial aspirations. Cellulase free xylano-pectinolytic microorganisms isolation from the cost effective and novel protocol. Experiments have been made to compensate commercial substrates with agricultural wastes such as citrus peel, waste paper, wheat bran, etc. Some alternatives are mentioned in the previous findings for the use of citrus wastes as substrate for fermentation processes. Citrus waste is basically the juice extraction residue. For the production of biogas, microbial biomass product, and ethylene, many references are applicable (Lane 1979; Lequerica and Lafuente 1977; Rodrfguez et al. 1985; Chalutz et al. 1983). A cost-effective and easily available agro-waste is pineapple waste, which is used as a solid-state support for the blended culture of *Aspergillus fumigatus* and *Aspergillus sydowii* at 35 °C. Optimization of processing parameters arose in higher pectinase production. According to Kutateladze et al. (2009), for the polygalacturonase production, *Aspergillus* spp. dominate. The yield of polygalacturonase in the submerged fermentation is higher than earlier reports, i.e., 5.8850 U/mL and 4.7745 U/mL. In India agro-waste utilization as substrates for pectinolytic enzyme production is essential due to its abundance. According to Bayoumi et al. (2008), the agro-waste usage for enzyme production is an effective approach to convert raw material to a useful material. Few

findings reported different substrates such as wheat bran, wheat straw, rice bran, sugarcane bagasse, sugar beet pulp, sawdust, tea waste, apple pomace, orange peel, etc. Worldwide, biodegradable wastes and their disposal have been a big environmental concern. Agricultural waste can be used to produce useful compounds that help control environmental pollution (Omojasola and Jilani 2008). One of the important biomass wastes is orange peels. According to the literature, orange production was figured to advance 66.4 million tons, representing a 14% increase within 12 years. Almost 60% of oranges are processed for juice production, and the rest is expressed as citrus-processing waste. During bioconversion of agroindustrial waste, breakdown of polymers to monomers is important to further convert it to ethanol and other products. Largely orange peel is made up of cellulose, hemicellulose, pectins, chlorophyll pigments, and other low molecular weight aggregates like limonene. In orange peels two major hydrolyzing polymers are pectins and celluloses. Fungi produce pectinase enzyme that can break down pectin to galacturonic acids. Fungi secrete pectinases and other enzymes into the culture media. Production of industrial enzymes is expensive in developing countries because production is from pure and polished substrates. Thus, locally cheaper substrates can be used for enzyme production.

7.8 Conclusion

Researchers are trying to find out cost-effective, renewable technology for sustainable development and for commercial viability. In this concern, production of pectinase has been reported by many researchers using agro-waste as substrate. Many studies on the pectinase production from distinct cheap and low-cost sources such as lemon peel, apple waste, rice husk, sugarcane bagasse, and wheat bran are accessible in the literature. In India sugarcane bagasse, rice husk, wheat bran, apple peel, and lemon peel are in abundance, so these wastes can be used as most preferable substrates for pectinase production. In this interest, different agricultural wastes used for the pectinase production have been disclosed by distinct researchers. Previous findings were limited on cheap production of enzyme instead of profitable screening of enzymes. But recent studies have revealed the use of pectin for the screening of enzymes also. During screening of pectinase enzyme, purified pectin usage will be too extravagant for partial enzyme activity assessment. For the screening of microorganisms, these expensive commercial substrates especially pectin have been replaced with agricultural wastes, i.e., citrus peel and wheat bran. This advanced approach will be possibly suitable in large-scale screening aspirations of microorganisms. As there is a need of bulk enzymes production at a cost-effective rate so to achieve this goal, cost-efficient and ecofriendly methods should be adopted. Out of different pectinases, most important are bacterial extracellular pectinases, as compared to plants, viruses, animal, and fungal extracellular pectinases. Thus, the objective of the present study is to develop an economical medium for the isolation of pectinolytic bacteria using agro-wastes instead of pure commercial substrates. Commercial substrates and agro-waste result are comparable.

So, agro-waste should be utilized for the production of microorganisms and for the generation of the enzymes which reduces the cost of production

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Gallic Acid (GA): A Multifaceted Biomolecule Transmuting the Biotechnology Era

8

Sunny Dhiman and Gunjan Mukherjee

Abstract

Gallic acid is a naturally occurring phenolic acid that is widely distributed across the plant kingdom in various plant parts such as leaves (bearberry), roots and bark (pomegranates and gallnuts). The present chapter reviews distribution and occurrence of gallic acid in nature with emphasis on its dietary sources and biosynthesis. Another focus of the chapter relates to the approaches for making gallic acid, including extraction from plants, acidic/alkaline hydrolysis of gallotannins and enzymatic hydrolysis of hydrolyzable tannins. This chapter provides detailed information about the worldwide manufacturers of gallic acid along with various approaches for detecting and quantifying gallic acid in a wide variety of biological matrices. Apart from these, information on versatile applications, various patents on gallic acid and its ester derivatives have also been provided. The present chapter also discusses current challenges and future outlook wherein several key areas have been highlighted that require extensive research investigations to make gallic acid a real utility in the biotechnology era.

Keywords

Gallic acid · Polyphenol · Biomolecule · Biosynthesis · Tannins

8.1 Introduction

Gallic acid is reckoned as one of the most valuable biochemicals in biotechnology era on account of versatile applications of this biomolecule in different fields. Gallic acid and its derivatives have been produced and reported to evince a number of

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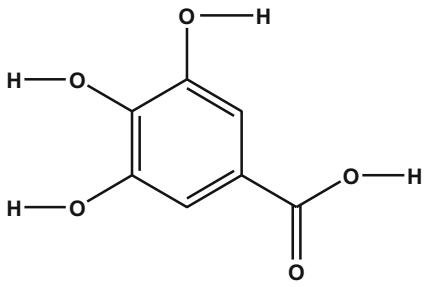

Fig. 8.1 Structure of gallic acid

biological and pharmacological properties. Gallic acid (3,4,5-trihydroxybenzoic acid) is a phenolic acid which naturally occurs in a vast variety of herbs and foods like blueberries, apples, flax seed and walnuts and in several types of processed beverages such as green teas, red wines, etc. Gallic acid also occurs profoundly in gallnuts, tara fruit pod, oak bark, teri pod cover, myrobalan, sumac and several other herbs and plants. Carl Wilhelm Scheele (1786) was the pioneer to discover the existence of gallic acid in plants (Fischer 1914). Gallic acid has been named so after oak galls which were historically used in preparation of tannic acid. Gallic acid occurs invariably as free acids, esters, catechin derivatives and as a part of hydrolyzable tannins. Gallic acid is a key precursor for various secondary metabolites especially gallotannins and ellagitannins (Haslam 1998; Grundhoefer et al. 2001). Gallic acid is a planar molecule comprising an aromatic ring which has a carboxylic acid group and three phenolic hydroxyl groups bonded to it in an ortho position with respect to each other (Fig. 8.1). This particular array of arrangement in phenolics is the principal determinant of their antioxidant potential (Sroka and Cisowski 2003). Three hydroxyl groups bonded to the aromatic ring in gallic acid structure are susceptible to getting oxidized whereby leading to generation of hydrogen peroxide, quinones and semiquinones (Severino et al. 2009). Gallic acid is a crystalline solid which is marginally colourless or slight yellowish in appearance. It has a molecular formula $C_7H_6O_5$ with molecular weight of 170.11954 g/mol. The melting point of gallic acid is 210 °C and disintegrates between 235 °C and 240 °C thereafter generating carbon monoxide alongside carbon dioxide. Gallic acid has a density of 1.69 kg/L (20 °C), pKa value of 4.40 and log P of 0.70 (20 °C). It is fairly soluble in water and various other solvents such as acetone, ether and glycerol and virtually insoluble in chloroform, ether, petroleum and benzene (National Institutes of Health [NIH] 2015).

An in-depth perusal of literature reveals that gallic acid and its derivatives have been produced and documented to possess several biological and pharmacological properties. They are broadly distributed in several plants and fruits in nature. Gallic acid and its derivatives are considered as extremely salutary owing to their vast utility in numerous pharmacological and industrial applications. Gallic acid has numerous therapeutic and industrial applications that range from anticancer, radioprotective, antiviral and antibacterial agent to utilization in the food industry, agriculture sector, cosmetics, photography industry, etc. Various esters of Gallic

acid such as propyl gallate, octyl gallate and lauryl gallate etc. possess immensely effectual therapeutic properties which includes anticancer, antiulcer, antiallergic, antiarteriosclerosis, antiangiogenic, antioxidative, antiviral, antibacterial, anti-inflammatory, anticarcinogenic and radioprotective agent etc. (Locatelli et al. 2013; Choubey et al. 2015). Owing to the vast range of applicability of Gallic acid and its esters; they are broadly utilized in cosmetics, in foods, adhesives, printing inks, photography, lubricants, dyes etc. (Li et al. 2011; Brewer 2011; Saeed et al. 2012). In addition, gallic acid is also used as a standard for determining the phenolic content by Folin-Ciocalteu assay. Apart from their broad spectrum of industrial and pharmacological applications, gallic acid and its esters are widely utilized by human community in several forms such as preservatives and additives in various food stuffs, etc. in direct or indirect ways. Broadly utilized food antioxidant additives, i.e. methyl gallate (MG), lauryl gallate (LG) and propyl gallate (PG), are produced using gallic acid. The structures of some of the important derivatives of gallic acid have been described in Fig. 8.2a, b.

A lot has been reviewed on pharmacological applications of gallic acid. Most of the research investigations have been carried out utilizing the therapeutic potential of gallic acid. There are certain reviews on methods of quantification of gallic acid (Fernandes and Salgado 2016) and pharmacological applications of gallic acid (Subramaniana et al. 2015; Nayeem et al. 2016; Choubey et al. 2018). However, a comprehensive review on various aspects of gallic acid has not been attempted yet. This chapter thus is an attempt to provide a broader perspective on various aspects of gallic acid. Here we will review distribution and occurrence of gallic acid in nature with emphasis on its dietary sources and biosynthesis. Another focus of the paper relates to the approaches for making gallic acid, including extraction from plants, acidic/alkaline hydrolysis of gallotannins and enzymatic hydrolysis of hydrolyzable tannins particularly the gallotannins. This chapter will also provide detailed information about the worldwide manufacturers of gallic acid along with various approaches for detecting and quantifying gallic acid in a wide variety of biological matrices. We will also be reviewing versatile applications of this valuable biomolecule and its ester derivatives in details. Apart from these, detailed information on several patents worldwide on gallic acid and its ester derivatives will also be provided. Lastly, we will discuss current challenges and future outlook wherein several key areas will be highlighted that require extensive research investigations to make gallic acid a real utility in the biotechnology era. The detailed information provided in this chapter would be worthwhile for scientific communities and researchers engaged in various sectors especially those working on different aspects of plant-derived phenolic compounds.

8.2 Distribution and Occurrence of Gallic Acid in Nature

Gallic acid is a naturally occurring phenolic acid that is widely distributed across the plant kingdom in various plant parts such as leaves (bearberry), roots and bark (pomegranates and gallnuts). Gallic acid is broadly distributed in plant kingdom

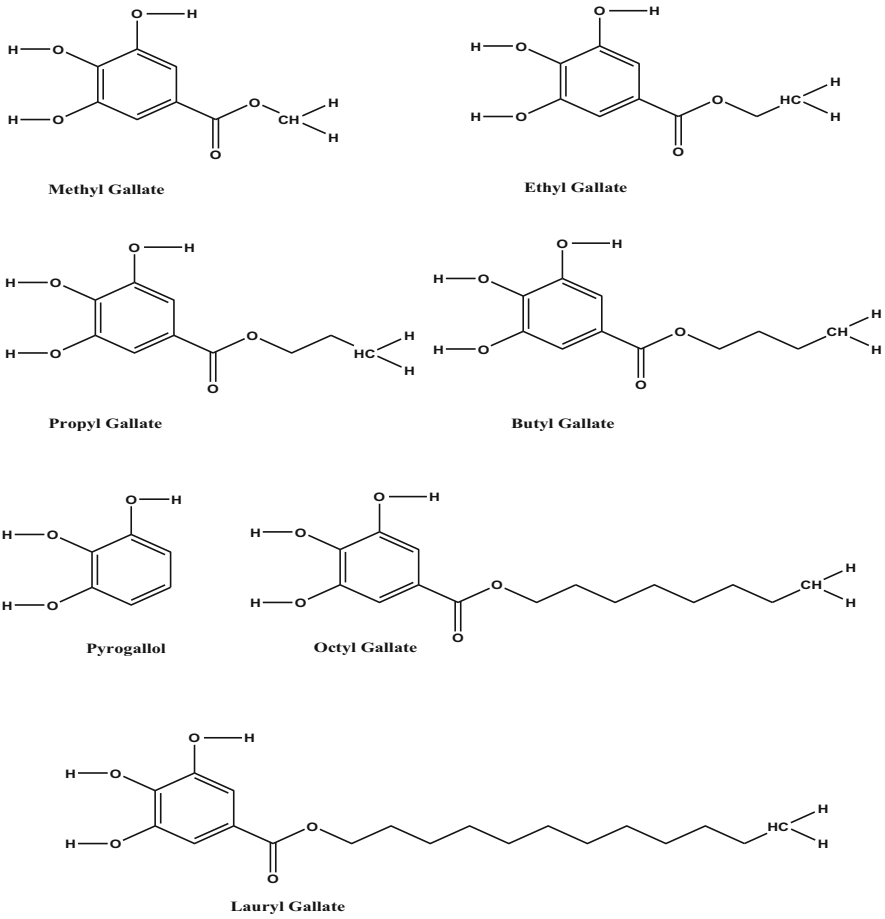

Fig. 8.2 **(a)** Important ester derivatives of gallic acid. **(b)** Esters of gallic acid with gallocatechin and epigallocatechin

and majorly occurs either in free form or as its derivatives in diverse foodstuffs like tea, pomegranate, blueberries, nuts, grapes, apple, walnut, wine, etc. Gallic acid exists in several plants in the form of free acids, esters, catechin derivatives and as hydrolyzable tannins (Karamac et al. 2009). Gallic acid occurs in plenteous amount in plant bark (*Quercus* sp.), fruit seed kernel (*Mangifera indica*), tamarind seed (*Tamarindus indica*), leaves (*Syzygium cumini*, *Phyllanthus emblica*), fruit rind (*Punica granatum)*, fruit pod (*C. spinosa*) gall (*Quercus*), needles *(Pinus*), honey, red wines, berries, various vegetables, etc. Distribution of gallic acid in nature covers various families of vegetable kingdom like Myrtaceae, Fabaceae, Anacardiaceae, etc. (Battestin et al. 2004; Santos and Mello 2003). Gallic acid is also found in fungi belonging to genera *Termitomyces* (Puttaraju et al. 2006). The occurrence of gallic

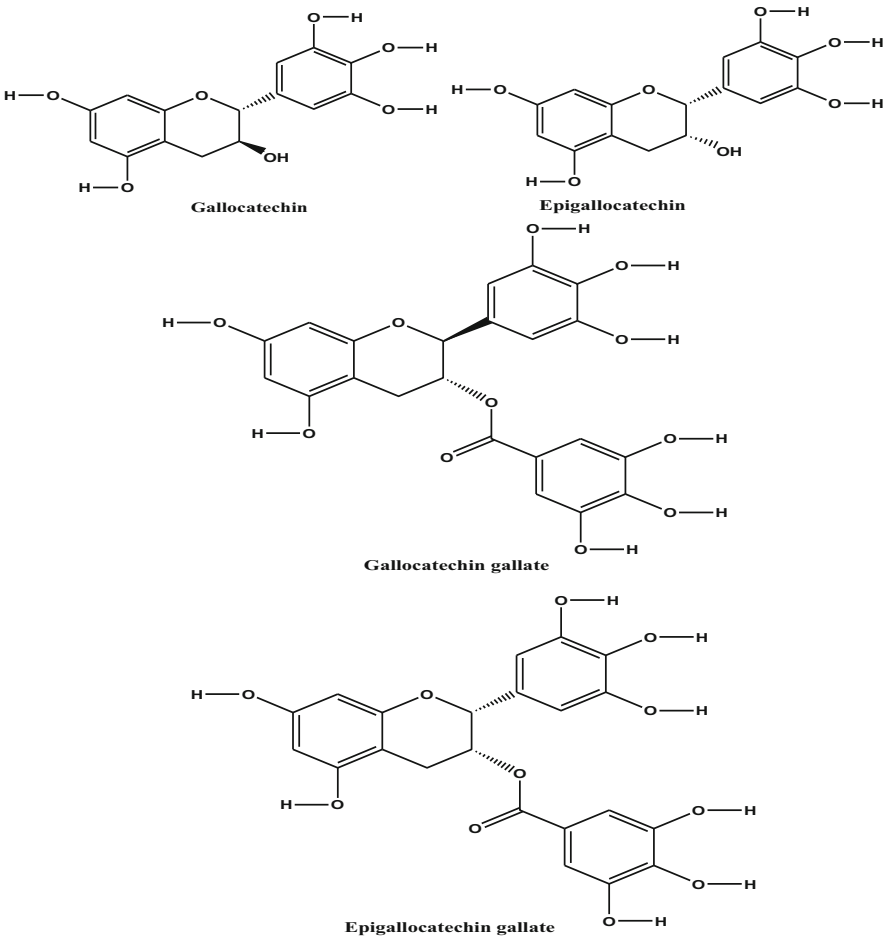

Fig. 8.2 (continued)

acid has been documented in several plants like *Vitis vinifera, Syzygium cordatum, Toona sinensis, Bridelia micrantha, Psidium guajava*, *Rhus typhina*, *Diospyros cinnabarina, Tamarix nilotica, Garcinia densivenia, Paratecoma peroba, Caesalpinia sappan, Rubus suavissimus*, etc. (Shahriar and Robin 2010). Various external factors like UV radiation, chemical stressors and microbial infections may also have an influence on the concentration of gallic acid present in different plant tissues.

8.3 Major Dietary Sources of Gallic Acid

Major dietary sources of gallic acid cover several vegetables like onion and black radish; fruits such as grapes, pomegranate and apple; nuts (walnut, cashew); and various beverage types (red wines, white wine, green tea, black tea, etc.) (Table 8.1). Gallic acid is a naturally occurring compound largely present in oak bark, sumac, tea leaves, tara pods, clove, teri pod, Turkish galls, Chinese galls, etc. Various eatables such as Indian gooseberry, blackberry, black raisin, strawberry, raspberry, cardamom, cuddapah almond, coconut, groundnut, white currant, black currant, vinegar, etc. contain significantly good amount of gallic acid. Gallic acid is one of the major components of grapeseed extract (GSE). Grapeseed extract has enormous antioxidant potential. Thus, it is reckoned to be tremendously effective against a number of diseased conditions (Veluri et al. 2006; Gonzalez-Abuin et al. 2014). Its consumption may have beneficial effect on conditions like high blood pressure, can improve blood flow, can enhance wound healing and may reduce the oxidation of LDL cholesterol which is a high-risk factor for heart disease. It may improve collagen and bone strength and may improve kidney function by providing protection against oxidative damages. Since grapeseed extract contains gallic acid as its major constituent, its consumption may inhibit the formation of clusters of beta-amyloid proteins

Table 8.1 Major dietary sources of gallic acid

Source	Gallic acid content
Beverages	
Red wine	95 mg/L
French wine and spirit	31–38 mg/L
White grape wine	1 mg/L
Semi-fermented tea	4500 mg/kg
Japanese green tea	2300 mg/kg DW
Chinese green tea	5200 mg/kg
Black tea	3200–3600 mg/kg FW
Fruits and vegetables	
Clove buds	175 mg/kg
Coconut	11.64 mg/kg
Groundnut	14.05 mg/kg
Fox nut	3.92 mg/kg
Black raisin	14.97 mg/kg
Cuddapah almond	16 mg/kg
Cardamom	6.41 mg/kg
Cashew nut	8.62 mg/kg
White currant	3–38 mg/kg
Blackberry	8–67 mg /kg
Black currant	30–62 mg/kg
Raspberry	19.38 mg/kg
Strawberry	11–44 mg/kg

DW = Dry wt., FW = fresh wt Compiled from (Rice-Evans et al. 1997; Tiwari et al. 2009; Tomas-Barberan and Clifford 2000)

in the brains of patients of Alzheimer's disease. Grapeseed extract also possesses strong antibacterial and antifungal properties. Its consumption may also protect the liver through its detoxifying activity. Several research studies have revealed that consumption of grapeseed extract can significantly inhibit the growth of common food-borne bacterial infections including *E. coli*, *Campylobacter,* Shiga toxins, etc. (Silvan et al. 2013; Zhu et al. 2015). Grapeseed extract (GSE) is also largely utilized in traditional medicine as a remedy for candida. Tea is reckoned as one of the wealthiest sources of gallic acid amidst various routine dietary foodstuffs. A great majority of foods rich in gallic acid have been utilized as natural remedies for several years. Native Americans and the early American settlers used to prepare aromatic tea using blueberries. The aromatic tea so prepared was used as a tranquilizer at the time of childbirth as well as an analeptic agent in purification of the blood (Ayaz et al. 2005). The above-mentioned benefits of dietary sources high in gallic acid outline its remarkable importance as an antioxidant and an immunity booster, emphasizing the inclusion of foodstuffs containing gallic acid in the routine diet for well-being of an individual thus leading to a better and healthier lifestyle.

8.4 Biosynthesis of Gallic Acid

Gallic acid is produced abundantly by fungi and plants (Werner et al. 1997). Owing to its immense importance as an antioxidant in numerous foodstuffs and beverages, the nutritional merit of several plants species and crops could be significantly ameliorated by controlling and influencing its synthesis and agglomeration (Muir et al. 2011). Thus, in this regard a vast number of research investigations have been documented pertaining to the explication of biosynthetic pathways of gallic acid. It could be produced either from phenylalanine or from an early shikimate intermediate, i.e. 3-dehydroshikimate (3-DHS) (El-Basyouni et al. 1964; Zenk 2014; Kato et al. 1968). Interestingly, the carboxylic group of gallic acid has been reported to be biosynthetically analogous to the carboxylic group of shikimate, rather than to the phenylalanine side chain (Dewick and Haslam 1968; Dewick and Haslam 1969; Werner et al. 2004). Thus, it emphasizes the formation of gallic acid from 3-dehydroshikimate either via direct dehydrogenation or through protocatechuic acid as an intermediate (Fig. 8.3) (Kambourakis et al. 2000; Li and Frost 1999). Werner et al. conducted retrobiosynthetic NMR studies with 13C-labelled glucose and oxygen isotope ratio mass spectrometry and revealed that synthesis of gallic acid occurs primarily via direct dehydrogenation of 3-DHS (Werner et al. 1997; Werner et al. 2004). Two distinct pathways could be operative in the same organism, i.e. via early shikimate intermediate and via one of the aromatic amino acids (Zenk 2014; Ishikura et al. 1984; Saijo 1983). It has been reported that dehydrogenation of shikimate is more predominant in juvenile leaves of *Rhus succedanea* for synthesis of gallic acid in contrast to the alternative phenylalanine pathway in old leaves (Ishikura et al. 1984). Shikimate dehydrogenase (SDH), a vital enzyme of the shikimate pathway, has been explored (Werner et al. 2004; Ossipov et al. 2003). Shikimate dehydrogenase catalyzes the reduction of 3-dehydroshikimate to shikimic

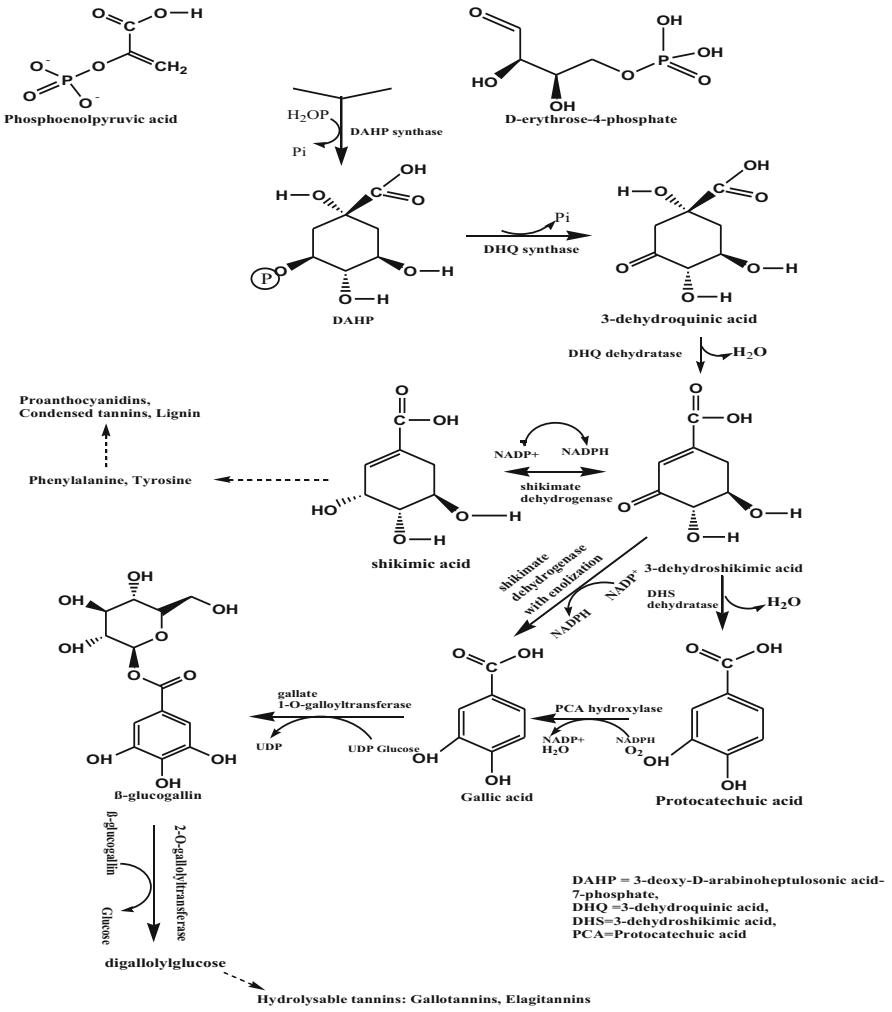

Fig. 8.3 Biosynthesis of gallic acid in plants

acid in the presence of NADPH. On the other hand, it catalyzes the oxidation of 3-dehydroshikimate to 3,5-didehydroshikimate which is a volatile compound undergoing a spontaneous rearrangement to gallate (Muir et al. 2011). Thus, shikimate dehydrogenase is a key enzyme responsible for the reduction of 3-DHS to shikimic acid. Shikimic acid is principally utilized in the synthesis of aromatic amino acids L-tyrosine, L-phenylalanine and L-tryptophan (Singh and Christendat 2006). Shikimic acid is also required in the biosynthesis of gallic acid.

8.5 Approaches for Gallic Acid Production

Gallic acid exists in plants in both free form and in bounded form as a component of hydrolyzable tannins. The hydrolysis of hydrolyzable tannins results in their depolymerization to release gallic acid and glucose or ellagic acid and glucose depending upon the type of tannin being hydrolyzed. Currently three approaches are used for making gallic acid.

8.5.1 Extraction from Plants

The extraction of gallic acid is of immense practical interest since it possesses numerous valuable biological characteristics. Gallic acid extraction from different plant parts is an established approach that primarily involves usage of solvents like water, ethanol, methanol, etc. for extraction. Swedish chemist Scheele was the pioneer to extract gallic acid from gallnuts around 200 years (Haslam 1986). So far gallic acid has been extracted from a vast range of plants. It has been extracted from a wide variety of sources that includes stem, bark, fruits, leaves, woods, seeds, pods, rinds, etc. The most widely used solvents for extracting gallic acid are methanol, ethanol and water. Gallic acid isolation has been reported from the methanolic extracts of plant leaves of *Tectona grandis*; methanolic extract of whole plant of *Bergia suffruticosa*; aqueous ethanolic extract of leaves of *Ceratonia siliqua*; methanolic extracts of wood, bark, fruits and leaves of *Casuarina equisetifolia*; and ethyl acetate soluble portion of the methanol extract of fruit pulp of *Terminalia chebula* (Sheetal et al. 2007; Nayeem and Karvekar 2010; Aher et al. 2010; Genwali et al. 2013). The general methodology of gallic acid extraction from various plant sources has been depicted in Fig. 8.4.

8.5.2 Acid/Alkaline Hydrolysis of Gallotannins

Gallic acid production can also be done by the hydrolysis of tannic acid and hydrolyzable tannins under acidic or alkaline conditions (Mukherjee and Banerjee 2003). This particular approach of making gallic acid is not viable from economic perspectives as well as on accounts of lesser yields and minimal purity profiles of gallic acid (Bajpai and Patil 2008). Moreover, this process of gallic acid production involves usage of significantly high concentrations of acid/alkali which eventually results in corrosion of containers being used throughout the process. This outlines the requirements of better, safer and efficient safety measures. One of the most adverse after effects of this approach is the release of toxic effluents that prove detrimental to the environment and eventually to the human beings and other forms of life (Banerjee et al. 2001).

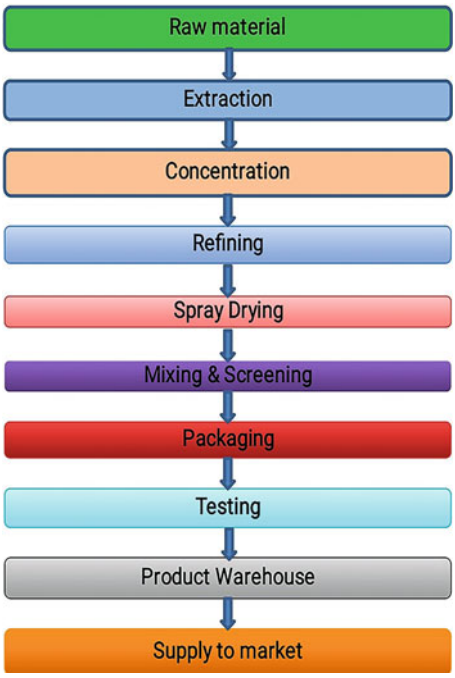

Fig. 8.4 General methodology of gallic acid extraction at industrial level from various plant sources

8.5.3 Enzymatic Hydrolysis of Tannins

It has been documented that tannins can be hydrolyzed using suitable enzymes especially microbial tannase (Aguilar and Gutierrez-Sanchez 2001). A great majority of tannin hydrolyzing organisms reported to date pertain to bacteria, fungi and yeast. Tannase from filamentous fungi pertaining to genera *Aspergillus* and *Penicillium* has been documented as the most widely utilized tannase worldwide for tannin hydrolysis (Belmares et al. 2004; Macedo et al. 2005). However, on the other hand, this is a well-established fact that bacteria can effectively disintegrate natural tannins as well as tannic acid (Deschamps et al. 1983). In recent years the reliance on microbial tannases for hydrolyzing the tannins has accelerated tremendously (Dhiman et al. 2018). The enzymatic approach of making gallic acid principally involves cleavage of ester and depside bonds in hydrolyzable tannins and gallic acid esters using tannase enzyme. The tannase-catalyzed hydrolysis of tannins particularly the gallotannins results in liberation of gallic acid and glucose molecules. Microbial tannase-catalyzed hydrolysis of tannins may overcome the drawbacks of other existing approaches of tannin hydrolysis. The advancements in molecular tools and techniques have enabled a better understanding of tannase structure and the underlying mechanism of its action. Both gallic acid and tannase production are interlinked to each other since tannase catalyzes the disintegration of hydrolyzable tannins thereafter giving gallic acid and glucose or ellagic acid and glucose depending upon the type of tannin being acted upon. Various sources of

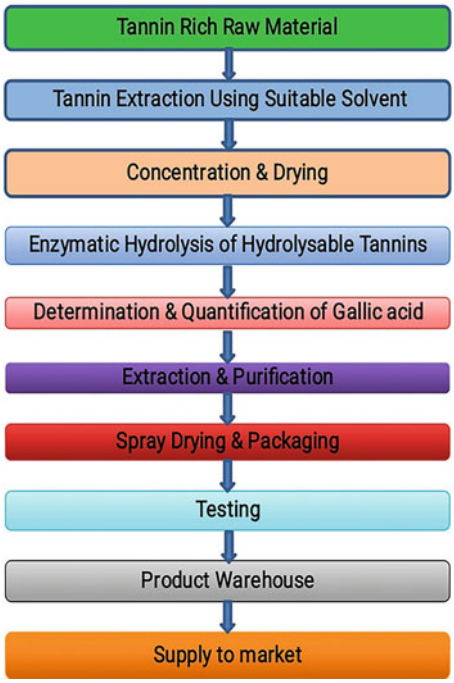

Fig. 8.5 General methodology of enzymatic hydrolysis of tannins for making gallic acid

hydrolyzable tannins principally utilized for gallic acid production are tara fruit pod (Pourrat et al. 1985), sumac leaves (Pourrat et al. 1987), gallnuts (Regerat et al. 1989), teri pod cover (Kar et al. 1999), myrobalan (Mukherjee and Banerjee (2004), *Cassia siamea* (Banerjee et al. 2007), *Larrea tridentata* (Trevino-Cueto et al. 2007), *Quercus infectoria* (Sarıozlu and Kıvanc 2009), *Acer ginnala* (Qi et al. 2009), mango seed kernel (El-Fouly et al. 2012), cashew testa (Lokeshwari 2016) and pine needles (Thakur and Nath 2017). With the advancements in biotechnology, efforts are being progressively made to replace the costlier raw materials with the cheaper and easily available agro-waste (e.g. pomegranate rind, tamarind seed, mango kernel, grapeseed, redgram husk, tea dust, cashew waste, etc.) and industrial waste (e.g. tannin waste from leather industries and other industries using plant-based constituents) for the production of valuable products of immense commercial importance like gallic acid. The general methodology of gallic acid production from various tannin-containing agro- and industrial waste-based substrates has been depicted in Fig. 8.5.

8.6 Scientific Perspectives on Gallic Acid Production

The production of gallic acid at industrial level is being done either through extraction or by hydrolyzing the tannins through acidic/alkaline treatment. The extraction of valuable chemical compounds from plants is difficult and

uneconomical due to their complex nature and interference by other compounds and impurities. On the other hand, hydrolyzing the tannins through acidic/alkaline treatment generates toxic effluents thus posing health hazards to humans as well as to the environment. The drawbacks of these methods clearly suggest the need for developing inexpensive, high-yielding and environment-friendly processes of manufacturing gallic acid. The hydrolysis of tannins using microbial tannase has gained pace in recent years (Dhiman et al. 2018). However, this is a well-documented fact that bacteria have the ability for efficient degradation of naturally occurring hydrolyzable tannins and tannic acid (Deschamps et al. 1983). Also, bacteria may prove as a source of thermostable tannase which might be able to work over a broad range of temperatures in terms of enzyme activity and stability (Beniwal et al. 2010; Beniwal et al. 2013). However, there are very few research investigations involving tannase and gallic acid production on fermenter level (Raghuwanshi et al. 2011). The enzymatic hydrolysis of hydrolyzable tannins especially utilizing bacterial tannase can overcome the shortcomings of the chemical methods because of better process control and optimization, ability to genetically alter the microbes, safety, reproducibility as well as credibility.

8.7 Gallic Acid Manufacturers Worldwide

The global annual demand of gallic acid is 8000 tons. However, the global consumption is expected to accelerate remarkably in the coming future on account of versatile applications of this biomolecule in diverse fields. China leads amongst the gallic acid manufacturers worldwide. Some of the gallic acid manufacturers from China and the world have been listed in Table 8.2 and Table 8.3.

8.8 Methods of Detection and Quantification of Gallic Acid

Several methods are available for quantifying gallic acid in a wide range of biological samples (Table 8.4). These include chromatographic, spectroscopic and electrophoretic methods along with others. All these methods have been developed and adopted worldwide to efficiently detect and quantify gallic acid in a great majority of samples to be analyzed.

8.8.1 Chromatographic Methods

Chromatographic methods of quantifying gallic acid include techniques like high-performance liquid chromatography, gas chromatography and thin-layer chromatography. They are most widely utilized for identifying and quantifying gallic acid in various matrices.

Table 8.2 Gallic acid manufacturers in China

Company	Product	Source/type	Part used	Methodology adopted	Form	Appearance	Grade
Dalian Sinobio Chemistry Co. Ltd.	Gallic acid	Grapeseed extract	Seed	Liquid-solid extraction	Powder	Light brown needle crystal	Industry grade
Hugestone Enterprise Co. Ltd. (Nanjing)	Gallic acid	Herbal extract	Seed	Solvent extraction	Powder	White fine powder	Food and medical grade
Xi'an Lyphar Biotech Co. Ltd.	Gallic acid	Chinese gall	Seed	Liquid-solid extraction	Powder	White fine powder	Medicine grade, food grade
Shaanxi Herbchem Biotech Co. Ltd.	Gallic acid	*Galla chinensis* (herbal extract)	Leaf	Solvent extraction	Powder	White powder	AAAAA
Xi'an Ceres Biotech Co. Ltd.	Chinese gall P.E. gallic acid powder	Chinese gall	Fruit	Solvent extraction	Powder	Brownish grey powder	Food and pharmaceutical grade
Shaanxi Undersun Biomedtech Co. Ltd.	Gallic acid	Gallnut	Seed	Solvent extraction	Powder	White powder	Food grade
Xi'an Sgonek Biological Technology Co. Ltd.	Gallic acid	Herbal extract	Seed	Solvent extraction	Powder	White powder	AAAAA
Xi'an Salus Nutra Bio-Tech Inc.	Gallic acid	Herbal extract	Fruit	Solvent extraction	Powder	Fine white to yellow powder	Food grade
Xi'an Sonwu Biotech Co. Ltd.	Gallic acid	Gallnut extract	Seed	Solvent extraction	Powder	White or yellow white powder	Food grade
Shaanxi Sangherb Bio-Tech Inc.	Gallic acid	Herbal extract	Seed	Solvent extraction	Powder	White crystal powder	AAAAA, Food and medical grade
Xian Plant Bio-Engineering Co. Ltd.	Gallic acid	Herbal extract	Other	Solvent extraction	Powder	Fine white to yellow powder	Food grade

Table 8.3 Gallic acid manufacturers and suppliers worldwide

Company	Country
A. B. Enterprises	India
Alpha Chemika	India
Suvchem	India
SS Synthesis	India
JPN Pharma Pvt. Ltd.	India
A & Z Food Additives Co. Ltd.	China
Nanjing Longyuan Natural Polyphenol Synthesis Factory	China
Hunan Linong Technology Co. Ltd.	China
Hebei Jobon Bio-Technology Co. Ltd.	China
Chemways Healthcare Pvt. Ltd.	India
Honyam Commercial Group HK Ltd.	China
Finance International Trade Co. Ltd.	China
Shanghai Conson Industrial Co. Ltd.	China
Samana Chemicals Pvt. Ltd.	India
Twinkle Chemi Lab Pvt. Ltd.	India
Shreeji Pharma International	India
Yuanda Chemical Company	China
Yihe-Chem Co. Ltd.	China
Foodchem International Corporation	China
Luxi Xiaoyuan Biotechnology Co. Ltd.	China
Dalian North Potassium Chlorate Works	China
Petrel Bio-tech Co. Ltd.	China
Xi'an Le Sen Bio-Technology Co. Ltd.	China
Xi'an Hao-Xuan Bio-Tech Co. Ltd	China
Xi'an Lyphar Biotech Co. Ltd.	China
Sinobio Chemistry Co. Ltd.	China
Hugestone Enterprise Co. Ltd. (Nanjing)	China
Cosmos Trading Group Ltd.	Hongkong
Victory Global Chemicals Company	Malaysia
Krun Thai Company Ltd.	Thailand
Derbiotec	Peru
Molinos Asociados SAC	Peru
Sociedad Mercantil (Exportacion) SA	Peru

8.8.2 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography is the most widely used technique for identifying and quantifying gallic acid amidst all chromatographic methods. Properties like high potency and magnificent resolution, enabling efficient segregation of individual components, have made HPLC a highly reliable and established approach for analysing gallic acid (Stalikas 2007). Since phenolic compounds are characterized by their high molecular weights and high polarities, this entails the use

Table 8.4 Detection/quantification of gallic acid in different biological matrices

Molecule	Production approach/ conditions	Yield	Detection/quantification method used	Reference
Gallic acid	Extraction from *E. camaldulensis* leaves using 50% ethanol for solvent extraction at 40 °C for 24 h	6 mg/gm	HPLC and FTIR	(Al-Ghanimi 2016)
Gallic acid	Bioconversion of tannins of powder of teri pod (*Caesalpinia digyna*) cover using *Rhizopus oryzae* at 32 °C, pH 4.5 for 72 h	90.9%	TLC, melting point analysis and NMR	Kar et al. (1999)
Gallic acid	Fermentation of tannins of myrobalan seed powder by *Aspergillus niger* MTCC 282 at 30 ± 2°, pH 5.6 for 90 min	7.68 mg / mL	Using a standard curve for quantifying gallic acid by measuring absorbance (spectrophotometrically) at 520 nm.	Lokeswari and Raju (2007)
Gallic acid	Fermentation of tannins from *Cassia siamea* by *Aspergillus aculeatus* DBF9 at 30 °C, pH of 5.5 for 36 h	6.8 mg/mL	Using specific extinction coefficient by measuring absorbance at two selective wavelengths of 254.6 and 293.8 nm	Banerjee et al. (2007)
Gallic acid	Fermentation of tannic acid (extracted from *Quercus infectoria* gallnuts) by *Aspergillus fischeri* MTCC 150 at 35 °C, pH ((3.3–3.5) for 37 h	0.56 g/L	Using specific extinction coefficient by measuring absorbance at two selective wavelengths of 254.6 and 293.8 nm	Bajpai and Patil (2008)
Gallic acid	Fermentation of tannins from *Quercus infectoria* (oak) by *Aspergillus niger* 3 and *Penicillium spinulosum* at 30 °C, pH (5.8–6.0) for 48 h	91.3% using *Aspergillus niger* 3 & 93.2% using *Penicillium spinulosum*	Using a calibration curve for quantifying gallic acid by measuring absorbance (spectrophotometrically) at 440 nm	Sarıozlu and Kıvanc (2009)

(continued)

Table 8.4 (continued)

Molecule	Production approach/ conditions	Yield	Detection/quantification method used	Reference
Gallic acid	Production of gallic acid by fungal endophyte *Phomopsis* sp. SX10 (isolated from *Acer ginnala*) in PDA liquid media at 28 °C for 7 days	200.47 mg/L	HPLC	Qi et al. (2009)
Gallic acid	Production of gallic acid from tannic acid using *Enterobacter cloacae* MTCC 9125 at 37 °C, pH 4.5 for 48 h	3.4 mg/mL	Using specific extinction coefficient by measuring absorbance at two selective wavelengths of 254.6 and 293.8 nm	Beniwal et al. (2010)
Gallic acid	Production of gallic acid from tannic acid using *Enterococcus faecalis* at 37 °C for 72 h	0.28 mg/mL	TLC and HPLC	Goel et al. (2011)
Gallic acid	Production of gallic acid from tannic acid using partially purified lyophilized tannase from *Bacillus sphaericus* at 4 °C, pH 5.0 for 24 h	90.8%	TLC and HPLC	Raghuwanshi et al. (2011)
Gallic acid	Production of gallic acid from tannic acid using *Lactobacillus plantarum* CIR1 by fermentation (for 24 h) using a gas-lift bioreactor	8.63 g/L	HPLC	Aguilar-Zarate et al. (2014)
Gallic acid	Production of gallic acid from tannic acid using *Bacillus subtilis* AM1 and *Lactobacillus plantarum* CIR1 for 12 h	2.416 g/L in case of *Bacillus subtilis* AM1 and 2.373 g/L in case of *Lactobacillus plantarum* CIR1	HPLC	Zarate et al. (2015)

(continued)

Table 8.4 (continued)

Molecule	Production approach/ conditions	Yield	Detection/quantification method used	Reference
Gallic acid	Production of gallic acid by using pine needles through solid state fermentation by *Penicillium crustosum* $AN_3KJ820682$ at 30 °C, pH 5.5 for 120 h	9.29 mg/g	Using a standard curve for quantifying gallic acid by measuring absorbance (spectrophotometrically) at 520 nm Detected using TLC, ATR-FTIR spectroscopy, HPLC	Thakur and Nath (2017)
Gallic acid	Isolation of gallic acid from methanolic seed coat extracts of *Givotia rottleriformis*	6 mg/g DW	RP-HPLC, IR, NMR, LC-MS	Kamatham et al. (2015)
Gallic acid	Extraction of gallic acid from rind of *Punica granatum* using ethanol and water as solvents in (40:60) ratio at 60 °C for 60 min	_	TLC, HPLC, FTIR, UV-spectra	Entessar et al. (2012)
Gallic acid	Solid state fermentation of tannin-rich powdered fruits of *Terminalia chebula* and *Caesalpinia digyna* pod cover powder by *Rhizopus oryzae* (at 30 °C, pH 4.5 for 60 h) and *Aspergillus foetidus* (at 30 °C, pH 45.0 for 72 h)	85.67% in case of *Rhizopus oryzae* and 90.48% in case of *Aspergillus foetidus*	Spectrophotometrically	Mukherjee and Banerjee (2004)
Gallic acid	Ultrasound-assisted extraction (UAE) of gallic acid from *Suaeda glauca* Bge. leaves at 51 °C, 19.52 mL/g (solid/liquid	6.21 mg/g	HPLC	Wang et al. (2016)

(continued)

Table 8.4 (continued)

Molecule	Production approach/ conditions	Yield	Detection/quantification method used	Reference
	ratio), for 42.68 min using 70% ethanol as solvent for extraction			
Gallic acid	Extraction of gallic acid from the wood of *Caesalpinia decapetala* at (65–70 °C) for 48 h using ethanol/water (70:30) as extracting solvent	17.85%	_	Pawar and Surana (2010)
Gallic acid	Isolation of gallic acid from the crude extract of *Polygonum capitatum* using a biphasic solvent system consisting of ethyl acetate-n-butanol-0.44% acetic acid (3:1:5)	51.5 mg from 1.07 g of the crude extract	Ultraviolet spectrometry, IR, LC/MS, TOF-MS, NMR	Chen et al. (2010)
Gallic acid	Ultrasound-assisted extraction (UAE) of gallic acid from pomegranate peel at 77 °C, solid to solvent ratio 1:40 for 5 min using 66% ethanol as solvent for extraction	1.13 to 3.58 mg/g DW	HPLC	Zivkovic et al. (2018)
Gallic acid	Extraction of gallic acid from the root of *Euphorbia hyloomla* by using water, 70% alcohol and acetone as extracting solvents	0.047%.	Ultraviolet spectrometry, HPLC	Guo et al. (2007)

DW dry weight

of a highly efficient separation method for segregation of compounds (Araptisas 2012). One of the major advantages of HPLC is its applicability in a broad range of arrays especially the herbal extracts and fermentation broth. The right selection of an appropriate method and solvent extraction is one of the most important aspects in using HPLC for analysing gallic acid. Various techniques like ultra-sonication, turbo-extraction, infusion and maceration are most widely used for extracting gallic acid from various arrays. Maceration and infusion are broadly used in comparison to other methods of extraction. Hydro-alcoholic solutions, a class of solvents, are most widely used for extraction in addition to acetone, water, methanol, etc. Ethanol and methanol are reported as most preferred solvents for extracting a vast range of phenolic compounds (Azmir et al. 2013). Reversed-phase columns (C-18 and C-8) are reported in majority of works involving separation of compounds using HPLC. Most widely utilized columns are of 250 mm. In contrary, the ultra-performance liquid chromatography (UPLC) utilizes relatively small columns like 100 mm and 150 mm for chromatographic separation of compounds. He et al. (2015) emphasized the use of large columns for arrays having relatively larger number of compounds despite the fact that much longer analysis time is required with such columns (He et al. 2015). UV-visible/diode array detector (DAD) is most preferred detection system in majority of HPLCs. However, in some analysis especially in pharmacokinetic analysis and UPLC system, mass spectroscopy may also be used (Sun et al. 2014). The most used wavelength range lies between 270 and 280 nm. However, some studies apply 210 nm in relation to primary absorption of gallic acid (Wang et al. 2000), while few others use 254 nm as a general wavelength for phenolic compounds (Arceusz and Wesolowski 2013).

8.8.3 Gas Chromatography (GC)

Gas chromatography is reckoned as a highly efficient approach for identifying and quantifying a great majority of compounds. Superb sensitivity and excellent detectability are some of the salient features of this technique (Penteado et al. 2008). Adequate volatility of the compounds is of the essential requisites for analysis using gas chromatography. In addition, the compound must not boil above 300 °C. Derivatization of samples in case of gallic acid enables a superior response of the detector front of the chromatographic system thus resulting in better and easier detection of gallic acid (Frias et al. 2014). Tor et al. (1996) derivatized samples before injection for analysing gallic acid using GC/MS. Nunes Selles et al. (2012) adopted derivatization method for analysing phenolic compounds, polyols and free sugars in mango (*Mangifera indica* L.) using GC/MS.

8.8.4 Thin-Layer Chromatography (TLC)

Thin-layer chromatography is used as a rapid and cheaper method for identifying a compound under investigation in biological matrices. Optimization of a most

suitable stationary phase and mobile phase combination is one of the most important aspects in analysing samples using TLC (Pedroso and Salgado 2013). Sharma et al. (1998) used a mobile phase comprising chloroform/ethyl acetate/acetic acid (50:50:1) for identifying gallic acid on silica gel plates. Braz et al. (2012) identified gallic acid in *Schinus terebinthifolius* Raddi and *Arctostaphylos* uva-ursi (L.) Spreng using toluene/ethyl acetate/methanol/formic acid (75:25:10:6) as a mobile phase and 1% $FeCl_2$ as visualizing agent. Dhralwal et al. (2008) devised a TLC method for analysing samples of *Bergenia ligulata* and *Bergenia ciliata.* An automated system known as high-performance thin-layer chromatography (HPTLC) or TLC densitometry was used as a reading system in place of a visualizing solution.

8.8.5 Spectroscopic Methods

Gallic acid exhibits absorption maxima at 272.5 nm with log ε 4.06 (NIH, 2015). This absorption results from the combined absorptions of main chromophore group (cluster benzoil), added to the three substitutions -OH (Pavia et al. 2013). Pawar and Salunkhe (2013) validated a method for quantification of gallic acid hydro alcoholic extract of Triphala churna at 273 nm. One of the major applications of UV-visible spectroscopy is the usage of gallic acid as a standard (Folin-Ciocalteu's method) for determining the total phenolic content in a sample. The results obtained are expressed in terms of gallic acid equivalents (Sanchez-Rangel et al. 2013; Balan et al. 2015). Near-infrared (NIR) spectroscopy is the application of spectroscopy for quantifying polyphenolic compounds in spectrum corresponding between 4000 and 10,000 cm^{-1}. NIR proved to be highly effective and economical in comparison to other methods like partial least squares (PLS) while comparing the data obtained in HPLC for predicting the total polyphenols in green tea (*Camellia sinensis* L.) (Schulz et al. 1999).

8.8.6 Capillary Electrophoresis (CE)

Capillary electrophoresis separates different ionizable compounds on the basis of differences of the migration front of an electric field. Gotti (2011) reported that capillary electrophoresis might prove a highly efficient approach for identifying compounds like gallic acid in different arrays. Arne Tiselius, a Swedish Chemist, was the pioneer to apply this technique in 1930 (Spudeit et al. 2012). Cartoni et al. (1996) applied capillary electrophoresis technique to separate gallic acid and other phenolic compounds present in Italian wines and several alcoholic beverages. Yue et al. (2006) also applied capillary electrophoresis technique to identify gallic acid and salidroside in *Rhodiola dumulosa*.

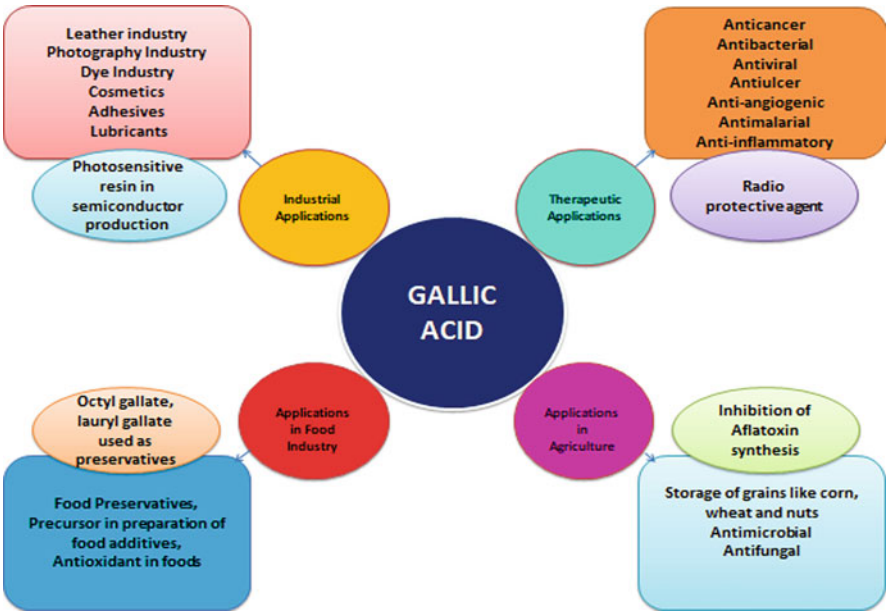

Fig. 8.6 Applications of gallic acid and its ester derivatives in different sectors

8.9 Applications of Gallic Acid and Its Derivatives

Gallic acid is reckoned as a molecule of immense significance owing its vast utility in various sectors ranging from food, agriculture and healthcare to cosmetics, photography, dye industry etc. (Fig. 8.6). Gallic acid and its derivatives have extended their utilization in diverse therapeutic and industrial applications (Table 8.5). This outlines the emergence of gallic acid as a molecule of stupendous market value in the last two decades.

8.10 Patents on Gallic Acid and Its Ester Derivatives

Several patents have been filed, published and granted for production, applications and other aspects of gallic acid and its ester derivatives (Table 8.6). The information collected herein has been sourced from various patents worldwide. The data presented here on different patents on gallic acid and its ester derivatives would be worthwhile to scientific community and researchers engaged in different sectors. A vast number of publications and patents on gallic acid and its derivatives in the past two decades give a clear indication of continuously expanding interest of researchers and scientific communities worldwide towards this valuable biomolecule.

Table 8.5 Applications of gallic acid and its ester derivatives in different sectors

Molecule	Type of application/ activity	Application details	Reference
Gallic acid	Antibacterial	Precursor for an antimalarial drug trimethoprim	Anderson et al. (1980); Ow and Stupans (2003)
Gallic acid	Antibacterial	Exhibits antibacterial activity against pathogens like *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*	Vaquero et al. (2007)
Gallic acid	Antimutagenic, Anticarcinogenic	Inhibition of tumour promoter-induced ornithine decarboxylase activity in mouse epidermis in vivo	Gali et al. (1991)
Gallic acid	Antioxidant, remote astringent	Provides protection to human cells against oxidative damage Can be used to treat albuminuria and diabetes Can be used as a remote astringent in case of internal haemorrhage	Abbasi et al. (2011)
Gallic acid	Antioxidant, anti-obese	Enhanced the levels of antioxidant enzymes and decreased the weight gain in rats fed with high-fat diets (HFD) and gallic acid	Hsu and Yen (2007)
Gallic acid	Antioxidant, antiulcer	Enhanced production of mucus rich in glycoproteins that helped in protecting the gastric lining	Sen et al. (2013)
Octyl gallate, lauryl gallate	Anticancer	Octyl gallate and lauryl gallate induced apoptosis of HL-60 cells	Htay et al. (2002)
Gallic acid	Antioxidant	Inhibition of amyloidogenic proteins such as β-amyloid, insulin and calcitonin that cause amyloid-associated disorders like Parkinson's disease, Alzheimer's disease, type II diabetes, prion diseases, etc.	Kocisko et al. (2003); Porat et al. (2006)
Epigallocatechin gallate, epicatechin gallate	Stabilizer of collagen in cosmetic fillers	Epigallocatechin gallate and epicatechin gallate proved to be effective in inhibiting the degradation of collagen-based cosmetic fillers by collagenase	Jackson et al. (2010)

(continued)

Table 8.5 (continued)

Molecule	Type of application/ activity	Application details	Reference
Propyl gallate, octyl gallate and lauryl gallate	Antioxidant/ stabilizer/food additive	Propyl gallate, octyl gallate and lauryl gallate are used in cosmetics and processed food products and food packaging materials for preventing oxidative damage and spoilage	Zhao et al. (2011)
Gallic acid	Enhancement of chromium uptake in tanning process	Beneficial in chromium uptake in chrome tanning process in leather industry	Ramamurthy et al. (2014)
Gallic acid	Ink industry	Used as a source material in ink industry	Nayeem et al. (2016)
Gallic acid	Antioxidant	Inhibition of degradation of DNA from oxidative damage by reactive oxygen species (ROS)	Ferk et al. (2011)
Propyl gallate	Antioxidant/ stabilizer	Provides protection to biodiesel oxidation	Chen and Luo (2011)
Gallic acid	Anti-tumour/ anticarcinogenic	A component of cosmetic products. Provides protection to the somatic cells against UV-B or ionizing irradiation; inhibits melanogenesis	Sawa et al. (1999); Su et al. (2013)
Gallic acid	Antioxidant	Prevents rancidity and spoilage of fats and oils. This facilitates use of gallic acid as a food additive in candies, chewing gums and several baked foods	Singleton (1981)
Gallic acid	Antifungal	Inhibits aflatoxin synthesis thus suitable for preservation of grains such as corn, wheat and nuts	Mahoney and Molyneux (2004)
Gallic acid	Hepatoprotective	Provides protection to hepatocytes against oxidative stress created because of hydrogen peroxide, carbon tetrachloride	Tung et al. (2009); Li et al. (2010)
Gallic acid	Neuroprotective	Used for treatment of neurodegenerative diseases like dementia, Alzheimer's disease, Parkinson's disease, epilepsy	Cho et al. (2011); Huang et al. (2012); Mansouri et al. (2013)

(continued)

Table 8.5 (continued)

Molecule	Type of application/ activity	Application details	Reference
Gallic acid	Antioxidant	Gallic acid in association with EDTA reduced motor and oxidative damages emanating because of lead poisoning. Provides protection to brain tissues against lead toxicity	Reckziegel et al. (2011)
Gallic acid	Antihyperglycaemic, antilipid peroxidative, antioxidant	Gallic acid showed antihyperglycaemic, antilipid peroxidative, antioxidant effects on streptozotocin-induced diabetic rats. Prevents diabetes by increasing the level of plasma insulin and glucose tolerance	Punithavathi et al. (2011); Prince et al. (2011)
Gallic acid	Anti-inflammatory	Plays antagonistic role against inflammatory cytokines like IL-10, IL-1β and TNF-α	Chen et al. (2014)
Gallic acid	Paper industry (improvement of kraft pulp)	Gallic acid in association with laccase improved the quality of kraft pulp thus leading to better tensile strength of paper	Chandra et al. (2004)
Gallic acid	Antiangiogenic	Gallic acid a major component of grapeseed extract (GSE) resulted in growth inhibition and apoptotic death of human prostate carcinoma DU145 cells	Veluri et al. (2006)
Gallic acid	Cosmetics (polymerization with syringic acid)	Development of a brown colour hair dye	Jeon et al. (2010)
Gallic acid	Antibacterial	Zein (Ze-GA) electrospun fibre mats containing gallic acid possess antibacterial activity and can be utilized as packaging materials in the food industry	Neo et al. (2013)
Gallic acid	Radioprotective	Protection against harmful gamma radiations	Nair and Nair (2013)
Gallic acid	Analytical reagent	Used in pharmaceutical industry and other analytical studies for estimation of the phenolic content of analytes expressed in terms of gallic acid equivalents (GAE)	Damiani et al. (2014); Roshanak et al. (2016)

Table 8.6 Patents on gallic acid and its ester derivatives

Title	Filing date	Publication date	Publication number	Assignee	Salient features	Reference
Use of gallic acid for stabilizing chrome-III against oxidation in chrome-tanned leather	06/19/2001	01/03/2002	WO/2002/000941A1	Cognis Deutschland GMBH and Co. KG (Henkelstrasse 67 Düsseldorf, 40589, DE)	The present patent describes the use of gallic acid as a stabilizer in stabilizing chrome-III against oxidation in chrome-tanned leather	(Candar et al. 2002)
Method of inducing sweetness by gallic acid and its applications	06/06/2001	06/06/2002	US200220068123	Justus Verhagen Scott Thomas R. Giza Barbara K.	This patent claims usefulness of gallic acid as a sweetener for foods, beverages and medicaments	Justis et al. (2002)
Cosmetics and pharmaceutical applications of gallic acid and gallic acid derivatives	03/18/2015	04/27/2017	US20170112737	Greenpharma (Orleans, FR) Bioalternatives (Gencay, FR)	This patent claims and discloses the applications of gallic acid and its derivatives in cosmetic composition for stimulating or repairing the barrier function of the epidermis; in pharmaceutical applications for treating the lesions caused by pathologies, such as Crohn's disease, eczema, atopic dermatitis, psoriasis, etc.	Philippe et al. (2017)
Process for the preparation of gallic acid by coculture	08/05/2004	12/16/2004	US20040253694	Banerjee Rintu, Mukherjee Gargi	A process for preparing gallic acid by fermenting tannin-rich mixed substrate with an inoculum comprising *Rhizopus oryzae* and *Aspergillus foetidus*	Banerjee and Mukherjee (2004)

(continued)

Table 8.6 (continued)

Title	Filing date	Publication date	Publication number	Assignee	Salient features	Reference
Polyester resins comprising gallic acid and derivatives thereof	04/30/2013	10/30/2014	US20140322641	Xerox Corporation (Norwalk, CT, US)	This patent discloses the use of gallic acid and its derivatives like gallic diol, gallic triol, gallic tetrol, etc. for making polyester resins for toner for imaging devices	Ke et al. (2014)
Angiogenic agents from plant extracts, gallic acid and derivatives	05/27/2004	02/08/2007	US20070031332	Inventors: Greenway, Frank L. (Baton Rouge, LA, US) Liu, Zhijun (Baton Rouge, LA, US) Woltering, Eugene A. (Kenner, LA, US)	This patent describes the antiangiogenic activity of gallic acid and its derivatives obtained from the extract of Chinese blackberry (*Rubus suavissimus*). It claims usefulness of gallic acid and its derivatives in treating diseases like obesity, rheumatoid arthritis, diabetic retinopathy, psoriasis, etc.	Greenway et al. (2007)
Enzymatic synthesis of gallic acid esters	07/19/1984	09/10/1986	EP0137601A3	Corning glass works	This patent describes an improved method for producing gallic acid esters by incubation of gallic acid with an alkyl alcohol or diol in the presence of immobilized tannase to obtain corresponding alkyl gallate ester	Weetall (1986)

Fungicidal mixtures based on gallic acid esters	05/04/2011	11/10/2011	WO/2011/138345A2	BASF SE (67,056 Ludwigshafen, DE) Rodriguez-Tello, Angel (Jardines de Cister 12, Seville, E-41014, ES) Stammler, Gerd (Korngasse 9, Dossenheim, 69,221, DE) KOPF, Alexander (Schustergasse 4, Speyer, 67,346, DE) Gold, Randall Evan (Birkenweg 3, Obrigheim, 67,283, DE) Klappach, Kristin (Kellereistrasse 16, Neustadt, 67,433, DE)	This patent describes the use of gallic acid esters in fungicidal mixture for protecting the crops from harmful fungi, insects and pests	Rodriguez-Tello et al. (2011)
Improved gallic acid synthase and method for producing gallic acid	03/10/2008	09/24/2009	JP2009213392A	Genaris Inc	This patent describes a new method of making gallic acid by using a microorganism expressing a protein thus making gallic acid from protocatechuic acid	Saito et al. (2009)
Application of gallic acid ester compound	07/07/2005	02/16/2006	JP2006045219A	Sumitomo Chemical Co Ltd	This patent describes the usefulness of ester derivative of gallic acid as a controlling agent towards the arthropods (e.g. termites) that create harm to the wood materials	Matsuo and Kubota (2006)

(continued)

Table 8.6 (continued)

Title	Filing date	Publication date	Publication number	Assignee	Salient features	Reference
Use of gallic acid esters to increase bioavailability of orally administered pharmaceutical compounds	03/01/2000	09/08/2000	WO/2000/051643A1	Avmax, Inc. (385 Oyster Point Boulevard, Unit 9-A South San Francisco, CA, 94080, US) Wacher, Vincent J. (1683 41st Avenue San Francisco, CA, 94122, US) Benet, Leslie Z. (53 Beach Road Belvedere, CA, 94920, US)	This patent describes usefulness of gallic acid esters (propyl gallate, methyl gallate, lauryl gallate, octyl gallate) in enhancing the bioavailability of orally administered pharmaceutical compound to a mammal requiring the treatment with the concerned compound	Wacher and Benet (2000)
Gallic acid as a laser direct thermal developer	10/01/1997	11/19/1998	WO/1998/052100A1	Imation Corp. (1 Imation Place, P.O. Box 64,898 Saint Paul, MN, 55164-0898, US)	This patent describes the use of gallic acid as a developing agent in thermographic elements used for imaging the thermographic materials using an infrared laser	Bjork and Philip (1998)
Plants transformed for elevated levels of gallic acid and methods of producing said plants	12/22/2004	07/28/2005	WO/2005/068625A1	The Regents of the University of California (the Office of Technology Transfer, 1111 Franklin Street, 12th Floor, Oakland CA, 94607-5200, US) Dandekar, Abhaya (39,766 Morning Dove Place, Davis, CA, 95616-9757, US) Muir, Ryann M. (848 Browning Circle, Woodland, CA, 95776, US)	This patent includes non-naturally occurring plants that contain elevated levels of gallic acid and various methods of producing such plants. This invention also provides certain genes and proteins that are useful in producing these plants	Dandekar and Muir (2005)

Stabilized gallic acid derivative and external preparation composition containing the same	04/30/1999	03/30/2007	JP3933344B2	Lion Corp	This patent describes the use of gallic acid derivatives as one of the components of a stabilized composition possessing properties such as antioxidant, elasticity imparting ability, skin bleaching, etc.	Mizushima et al. (2007)
Silver nanoparticle coated with gallic acid or its derivative	03/14/2008	10/01/2009	JP2009221505A	Dowa Electronics Materials Co Ltd	This patent describes the use of gallic acid derivatives like propyl gallate, octyl gallate, dodecyl gallate, etc. in creating novel silver nanoparticles possessing strong affinity towards various organic media	Sato et al. (2009)
Antimicrobial compositions comprising a natural agent selected from gallic acid, eucalyptol, naringin, a jasmonic acid compound and any combination thereof	07/18/2005	06/29/2006	WO/2006/068665A1	Kimberly-Clark Worldwide, Inc. (401 N. Lake Street, Neenah, WI, 54956, US) Greene, Sharon Linda (235 Della Smith Lane, Canton, GA, 30114, US) Huang, Yanbin (1288 East Hillsdale Blvd, Apt. 217 Foster City, CA, 94404, US) Huang, Lei (2473 Winsley Place, Duluth, GA, 30097, US) Weart, Ilona F. (506 Drifton Way, Woodstock, GA, 30188, US) Yang, Shu-ping (430 Fieldstone Landing,	This patent describes the use of gallic acid as one of the key components of an antimicrobial composition for treatment of fungal and yeast infections	Greene et al. (2006)

(continued)

Table 8.6 (continued)

Title	Filing date	Publication date	Publication number	Assignee	Salient features	Reference
				Alpharetta, GA, 30005, US) Malik, Sohail (150 West Park Drive, Apt. 701 Athens, GA, 30606, US) Johnson, Robert B. (1234 Rodrick Drive, Marietta, GA, 30066, US)		
Biocatalytic synthesis of galloid organics	02/23/2001	09/27/2001	WO/2001/071020A2	Board of Trustees Operating Michigan State University (East Lansing, MI, 48824, US) Frost, John W. (1621 Dobie Circle, Okemos, MI, 48864, US)	This patent describes a bioengineered synthesis scheme for producing gallic acid from a carbon source in the host cell by transforming the host cell (*E. coli*) with a gene encoding an enzyme capable of converting 3-dehydroshikimic acid to protocatechuic acid and a gene encoding an enzyme capable of catalyzing the hydroxylation of protocatechuic acid to gallic acid	Frost (2001)
Incorporation of flavan-3-ols and gallic acid derivatives into lignin to improve biomass utilization	04/19/2011	04/19/2012	US20120094330	Grabber John H. Ralph John	This patent describes the use of gallate esters of mono- and disaccharides as polymerizable monomers in method of producing modified lignin	Grabber and Ralph (2012)

Controlling agent of hardly controllable soil borne disease by gallic acid-related material, and method for controlling hardly controllable soilborne disease by using the same	06/16/2008	12/24/2009	JP2009298736A	Tokyo Univ of Agriculture Technology Okinawa Pref Gov	This patent describes the use of methyl gallate as one of the key ingredients of a controlling agent for hardly controllable soilborne disease. Potent antimicrobial property of methyl gallate is utilized here for controlling hardly controllable soilborne disease	Natsume et al. (2009)
Stable catalyst solution for electroless metallization	08/17/2012	02/20/2013	EP2559786A1	Rohm and Haas Electronic Materials, LLC (455 Forest Street, Marlborough, Massachusetts 01752, US)	This patent describes the use of gallic acid or its derivatives as a stabilizing agent of an aqueous catalytic solution comprising nanoparticles of metals like silver, gold, palladium, etc. and using this aqueous catalytic solution for electroless metal plating	Milum et al. (2013)

8.11 Final Remarks and Future Outlook

There is a need for diversifying substrates as well as microorganisms with a view to obtain more efficient gallic acid production. Dearth of information on exact metabolism of tannins limits the fermenter scale hydrolysis of tannins. Thus, extensive research studies would be required to get an in-depth knowledge of exact tannin metabolism. There is a need to search out novel strains especially the ones having ability to withstand high tannin concentrations which otherwise proves detrimental to microbes at higher concentrations. Chromatographic methods such as HPLC and GC are well-established approaches for quantifying gallic acid and other phenolic compounds in different biological matrices. However, more and more research investigations would be required to explore the applications of lesser used methods like capillary electrophoresis and IR spectroscopy in quantifying phenolic compounds. Apart from the facts listed above, extensive research studies would be required for exploration of novel applications of gallic acid and its derivatives in the future. Moreover, there is a need to establish better process controls to facilitate fermenter scale production of both tannase and gallic acid. This will make fermentation processes a real utility in meeting the demand of gallic acid and to overcome the shortcomings of existing methods for making gallic acid. In the near future the enzymatic approach of making gallic acid may become a trendsetter and set an optimistic baseline as a highly advantageous approach in comparison to other methods of making gallic acid.

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Role of Metagenomics in Plant Disease Management

9

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Abstract

Metagenomics employs present-day genomics mechanization to microbial communities in their innate habitats, omitting the obligation of culturing. Assembly of metagenomics sequence components is an arduous step, which involves quality check, assembly, binning, mapping, re-assembly, gene annotation and visualization. Numerous metagenomics scrutiny conduits along with visualization means are being refined from time to time to aid aforementioned process. This chapter furnishes a compendium of metagenomics applications in crop improvement via understanding and mitigating plant diseases through their appropriate management and control. Here, the role of metagenomics for plant disease management is illustrated with suitable examples for understanding microbial systems and microbiomes, plant-microbial interactions, disease diagnostics and phytopathology studies. Also, applications of metagenomics in isolation of novel microbial species for disease control, production of protective compounds for exogenous application and plant breeding for disease resistance and for production of disease-resistant genetically modified crops are elaborated. The chapter will be efficacious for students and researchers involved in plant stress and genomic studies and also to the coterie of scientists who have discerned the potentiality of metagenomics and are traversing the strategies involved.

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Disease resistance · Metagenomics · Phytopathology · Plant-microbe interaction

9.1 Introduction

Blooming and disease-free plants are delightful, grow well and have high yield. Being sessile, plants remain healthy as long as their surrounding supports normal growth and development. Typical sources that cause plant disease include biotic as well as abiotic factors. Abiotic factors comprise noxious degrees of temperature, radiation, pH, pollutants, heavy metals, etc., whereas biotic agents include living pathogens that cause diseases such as bacteria, fungi, viruses, viroids, mycoplasmas, protozoa and nematodes apart from ectoparasites like insects, mites, etc. In several ways, humans have afforded heavy losses due to plant diseases. Therefore, this compelling stage craves for refurbishing our traditional agricultural practices with enhanced contemporary techniques, touching the genetic regulatory controls to build up the crops' ability to confront, escape and endure pathogens, curtailing production concerns of the agricultural zone (Syed and Tollamadugu 2019). All plant diseases result from intercommunication among the host, the pathogen and the environment. Accordingly, plant diseases can be restrained by influencing one or more of these factors by invading sensitive zones in the phytopathological cycle. To achieve this, faultless interpretation of disease is crucial to spot the pathogen. Also, profound knowledge of the disease cycle, along with factors that influence the cycle, is essential to adequately manage any plant disease.

The antiquity of plant disease management is quite ancient. Humans invested in various control measures, viz. chemical, physical, cultural, etc., for crop protection without even knowing causative agent of the disease. Natural resistance of plants to varied forms of pathogens and diseases was perceived in the nineteenth century (Biffen 1905) which uplifted the paradigm from only 'crop protection' to 'protection together with crop improvement' through breeding for disease-resistant plants. Better understanding of classical plant genetics, host resistance and rapid improvement in research technology consequently contributing towards genomics revolution in the early twentieth century has successfully helped in controlling plant pathogenesis. This advancement at molecular level has swiftly elevated our understanding of the processes and components substructuring disease development and host-pathogen interaction. It has also added novel and efficient biomarkers for quick disclosure of the root cause. Addition of whole genome sequence databases along with next-generation sequencing (NGS) has strengthened genomic tools for managing plant diseases in more efficient and productive way. NGS has made achievable the metagenomics analysis of microbial colonies linked with plants' health. Whereas a genome equates to the entire genetic sketch of a lone individual, metagenomes speak for the genetic component of an integrated microbial community.

Metagenomics is acknowledged as megagenomics, environmental genomics, community genomics and ecogenomics too (Dupré and O'Malley 2007). It is

interpreted as the direct genetic analysis of genomes contained within an environmental sample. Metagenomics is an emanating branch where the potential of genomic analysis is enforced to integrated communities of microbial species, circumnavigating classical method of isolation and culture of individual microbial species. This allows examination of microbial fauna which was previously unreachable since only less than 1% of microbial species present on Earth can be cultured.

For metagenomics analysis, analyst fetches a sample from an appropriate habitat and does a mass isolation of the DNA from all the microbes in the specimen. Progressively, researchers are also deriving RNA or protein from the collective microbial species in the sample. Routinely, once the DNA/RNA is extracted from a sample, bacterial vectors are induced to intake and replicate the DNA/cDNA, thereby creating a genomic or cDNA library containing bits of all the microbial genomes present in the sample. Advanced sequencing technologies expedite sequencing DNA directly from a sample, which simplifies the process by sidestepping the establishment of a library. In sequence-based metagenomics, analysts converge on finding the full genetic sequence, while in function-based metagenomics they explore the products that microbes in a community can generate. Fundamentally, the accustomed stages concerned in a metagenomics projects are sequencing, processing and analysis. Processing involves reuniting the myriad of sequencing reads, annotating the assembled contigs to designate its role and accrediting contigs to distinct taxonomic group. Analysis is related to exploring the utilitarian and molecular assortment of microbial communities. Metagenomics analysis can be categorized into four sections depending upon diverse scanning approaches: (a) shotgun assay adopting mass genome sequencing, (b) genomic venture-impelled strategies devised to inspect definitive microbial activities, (c) genomic sequence analysis utilizing phylogenetic or functional gene expression, and (d) NGS for elucidating whole gene load in microbial community samples (Neelakanta and Sultana 2013).

Profuse schemes can be utilized for investigation of metagenomics shotgun data. Programs such as FastQC (Andrews 2017), Cutadapt (Martin 2011), BBDuk (http://jgi.doe.gov/data-and-tools) and Trimmomatic (Bolger et al. 2014) are used for controlling the quality. The reads can then be put together towards protracted adjoining sequences termed as contigs or carried straight to taxonomic classifiers. Metagenomics categorization resources equate sequences which are customarily reads or assembled contigs across a database of microbial genomes to label the taxon of every sequence. Genome assembly is a confronting issue, even for secluded genomes (Nagarajan and Pop 2013). Clustering of blended sample with profuse species in bizarre prosperity is much greater perplexing and necessitates appropriate assembly programmes. Short-read metagenome assemblies are usually hugely disintegrated by virtue of low coverage and interstrain variation. Binning algorithms tackle it and arrange contigs or scaffolds from the identical or intently analogous microbes. Binning algorithms include Meta-IDBA (Mahé et al. 2014), AbundanceBin (Callahan et al. 2016a), MetaVelvet (Callahan et al. 2016b) and MetaCluster (McMurdie and Holmes 2013; Siegwald et al. 2017; Oulas et al. 2015). Succeeding to binning, reads can be mapped back to the bins, and each bin

can congregated to generate lengthy contigs. Expert assemblers can be used for reassembly. Verification of the assembly and binning is a crucial stage in metagenomics genome refurbishment. MetaQUAST (Koren and Phillippy 2015) calculates genome statistics of assemblies and, by aligning with reference genomes, can document the quantity of incongruities. An exhaustive pipeline for assembly and annotation of metagenomics samples is MetAMOS (Treangen et al. 2013). Metagenomics has wide applications in plant disease management, starting from understanding the microbiomes up to get deeper insights into plant microbial interactions, disease diagnostics, phytopathology, disease control, disease forecasting, etc. This chapter elucidates the detailed account of these applications along with future prospects.

9.2 Role of Metagenomics in Understanding Microbial Systems and Microbiomes

Bulk data from metagenomics analyses empowers environmental microbiologists to portray microbe populace with higher details. Divergent microbes collected from various locales do not have a sure common history together, but can commingle because of their association with the reciprocal physio-chemical traits of their surroundings. Also, some microbial communities can be linked with each other in the collected samples, recognizing that individual taxa needs to break up from those partnerships through a mechanism comparable to genetic recombination (Garrett et al. 2012). Utilization of metagenomics methods to reveal diversity in microbial taxa has been reviewed by Neelakanta and Sultana (2013). Similarly, Myrold et al. (2014) have reviewed the power of metagenomics techniques for grasping soil microbial mechanisms.

9.3 Role of Metagenomics in Understanding Plant-Microbial Interactions

Flora and soil form mutually architect the organization and job of microbial taxa in the root zone. Individual plant variety is occupied by explicit microbial residents. Several phytopathogenic entities have simultaneously evolved with vegetation and demonstrate an elevated level of host specificity (Raaijmakers et al. 2009). Yet, until today, the genetic footing of this specificity is barely inferred. Metagenomics opens wide span area to unravel the molecular basis of host specificity. Another illustration is legume-rhizobia symbiosis, which is extremely explicit (Long 2001). A plant species conditional configuration of fungal and bacterial species which were found to have hostile behaviour against a phytopathogenic fungus, *Verticillium dahlia* Kleb. was reported by Berg et al. (2006). Metagenomics examination of biomolecules right away isolated from soils present in the root sector provided a prospect to analyse an extensive gamut of microbes inhabiting the root zone. Abdelfattah et al. (2016) had conducted metagenomics analysis of fungal diversity

on strawberry plants. They identified the structure of the fungal populations allied with discrete strawberry parts. Testimony and computation of this information can be utilized to understand complex interactions taking place among plants and occupant fungal diversity. Their documentation on comparative profusion of fungal microflora on various organs of the plant can significantly help in grasping the cause and regulation of complicated phytopathology. Such studies have prospective applied role in management of phytopathological conditions.

9.4 Role of Metagenomics in Phytopathology Studies

The microbial ecology of individual plant shapes a web which can be reflected by coevolution models (Cardinale et al. 2015). The tenacity and assembly of the system is crucial for the incursion of pathotypes. Microbes can further impede the introduction of disease-causing genes by tarnishing them through quorum sensing else the phytopathogens have to wrestle with other microbes for plant possessions (Friesen et al. 2011). Also, microbial species can straightforwardly effects plant's security response by emitting hostile biomolecules inside plant cells or on the exterior side of the plant (Fravel 1988). The study of plant microbiome data has engendered a model transferal in interpreting its role in disease development, and it has superabundant importance for phytopathological control and management.

Additional facet connected to the impact of farming exercises on soil and plant microbial ecology is emulated by the disease-suppressive soil phenomenon described as 'soils in which plants do not suffer from certain diseases or where disease severity is substantially reduced even though a virulent pathogen is present and the host plant is susceptible to the disease' (Weller et al. 2002; Haas and Défago 2005). Natural abolishment of soilborne ailments is an affair of activeness and configuration of soil microbial structure which can be methodically studied with the help of metagenomics. It was exhibited many years back that disease-suppressive characteristics of soil were mostly persuaded by enduring harvesting of wheat and potato monoculture piloting to accumulation of host-specific microbial population (Whipps 1997). This property can be exploited to produce disease-free plants in the field by understanding the host-specific microbial community through metagenomics analysis and thereafter its establishment in farmer's fields. The role of metagenomics in phytopathological studies of different microbes is explained below.

9.4.1 Bacterial

Symptoms of an infected plant by bacteria are very conspicuous, including wet rot, yellowing, dwarfism, chlorosis, necrosis, hypertrophy, hyperplasia, etc. Some of these symptoms are unapparent till the invasion is completed or the total plant is lost. Thereby, it is imperative to conduct a metagenomics study for detection of such diseases. da Silva et al. (2014) used olive knot as an exemplary disease to learn the

function and collaboration of coevolving bacterial populations in disease inception and development. In olive knot some of the bacterial species which does not cause any plant disease like *Erwinia toletana* coincides with the pathogen *Pseudomonas savastanoi* pv. savastanoi. Investigations have showed that the pathogenic as well as the non-pathogenic species coexist in the olive knot, and this adjacent juxtaposition perhaps has promoted swapping of bio-metabolites.

An endophytic bacterium is a division of endosymbiotic microbial taxa that endure in inner tissues of ostensibly fit host plants (Schulz and Boyle 2006). The endophytes help in making the nutrients available and, after that their uptake, boost biotic and abiotic stress resistance (Ryan et al. 2008; Hamilton et al. 2012). Their plant disease resistance boosting properties are connected with their qualification to generate a broad array of biocontrol agents, such as antibiotics, which can hinder the development of phytopathogens (Christina et al. 2013; Brader et al. 2014; Wang et al. 2014). Metagenomics analyses have shown that endophytes also provoke a concealed biotic stress defence procedure, i.e. induced systemic resistance (ISR), which bestows an intensified height of shield to a wide spectrum of phytopathogens (Pieterse et al. 2014).

9.4.2 Fungal

Fungal pathogens are competent of producing unspecified physiological change in plants. Inside kingdom of fungi resides several important groups such as rust, blast and mildews that do not grow in culture medium. Metagenomics, through the analysis of nucleic acid sequence, holds pledge to provide exhaustive information on the phylogeny of fungi. Utilizing metagenomics techniques, Hjort et al. (2014) had developed a clone from a soil-suppressive to club root disease on *Brassica oleracea* yielding the antifungal chitinase Chi18H8. Chapelle-Pineau et al. (2015) have combined metagenomic and meta-transcriptomic approaches to determine the transcriptional modifications in the rhizobacterial population of *Beta vulgaris* plant in *Rhizoctonia*-suppressive soil faced with the fungal phytopathogen. They noticed that after introduction of the pathogen, stress-linked genes were upregulated in rhizobacteria belonging to different families like *Sphingobacteriaceae*, *Alcaligenaceae*, *Cytophagaceae*, etc. Fusarium root disease in forest nurseries is one of the suitable models of the efficacy of metagenomics-related diagnostics. *Fusarium oxysporum* is morphologically indistinguishable from *F. commune* which is a destructive phytopathogen that causes damping-off in the conifer, *Pseudotsuga menziesii*, when in fact *F. oxysporum* is allegedly non-pathogenic (Stewart et al. 2006, 2012). Furthermore, as per Dumroese et al. (2012), *F. oxysporum* isolates have biocontrol ability to defend plantlets from damping-off disease triggered by *F. commune*. Metagenomics-founded diagnostics is the only existing technique to differentiate these two fungal species.

9.4.3 Viral

The diagnosis of plant viral pathogens is important because diseases caused by them occur quite frequently. Metagenomics sequencing of a plant infected by viruses involves the sequence of all microbes present, isolation of nucleic acid (mostly RNA) from unhealthy plants and creation of cDNA by random amplification method and decisively the sequence of possible plant pathogens. In a metagenomics study, it was found that the sequencing of cDNA obtained, derived from full RNA isolated from *Solanum lycopersicum* disease-ridden with the virus PepMV, a 97% identification of the viral genome sequence was achieved (Adams et al. 2009). Metagenomics-type inspection-seeking virus multiplicity in wild plants revealed that visible symptoms do not appear in wild plants infected with viruses (Stobbe and Roossinck 2014).

Maize lethal necrosis (MLN) is instigated by a synergistic coinfection of *Maize chlorotic mottle virus* (Nutter et al. 1989). Wamaitha et al. (2018) distinguished viruses related to MLN in Kenya by metagenomics investigation. Recognition of nucleic acid sequence directly derived from the site of infection is not explicit proof that the given microbe is the infecting agent. For instance, Adams et al. (2009) identified the full viral genomic sequence of a disease-causing virus, i.e. *Cucumovirus* through metagenomics study. This data is persuasive evidence that a transmissible disease with virus-like symptoms was associated with the occurrence of the *Cucumovirus* total genome reported in the infection site. Evidently, virus entities were not detected nor the disease was re-established in the primary host featuring that the virus existence can only be verified by metagenomics sequence exploration. Therefore, metagenomics will extend a state-of-the-art challenge for taxonomy and job of plant pathogens in phytopathology (Studholme et al. 2011).

9.5 Role of Metagenomics in Plant Disease Diagnostics

Metagenomics coupled with next-generation sequencing can spot plant diseases quickly to an eminent taxonomic rank without necessitating slight erstwhile information of the host or pathogenic organism. A metagenomics methodology to diagnose disease caused by phytopathogen presents the likelihood of conquering botheration of phytopathogen projection affiliated with counterpart diagnostic techniques like PCR and non-specificity connected with conventional probing methods (Adams et al. 2011). In the field of diagnosis, taxonomic grouping of sequence reads is principally paramount for diseases whose aetiology is obscure or whose symptoms may possibly be mimicked by various species of virulent entities (Bernardo et al. 2013). In the course of time, new phyto-viruses have been detected by metagenomics approaches (Al Rwahnih et al. 2009; Roy et al. 2013).

Apprehension for plant biosecurity stimulated the advancement of e-probe diagnostic nucleic acid analysis (EDNA; Stobbe et al. 2013). For plant protection, it is crucial to be conscious that peculiarly precarious microbes should be absent in

supplies traded across borders or through internal bioterrorist attacks (MacDiarmid et al. 2013; Kim et al. 2002). Metagenomics offers an excellent microbial forensics ability to defend against such bioterrorism (Fletcher et al. 2008; Fletcher et al. 2011).

Plant diseases where multiple pathogens are engaged in the infectivity are generally designated as 'complex' since their identification and consecutive monitoring are extra sophisticated. Corresponding syndromes crop up as an outcome of a network that includes a wide range of microbial interactions (Lamichhane and Venturi 2015). Monoculture inoculations are regularly executed to gauge the pathogenicity behaviour of a particular phytopathogen. Thus, our expertise of their likely synergism that directs to amplified disease acuteness is meagre. It is probable that synergism between distinct pathogens steering to brutal pathology symptoms happens frequently than anticipated (Begon et al. 1986). Such synergistic interfaces in plants are of pivotal attention for comprehension of microbial pathogenesis and consequent evolution of effective disease control strategies. Judgement as well as control of multifarious diseases can be protracted consequential to substantial production losses. In the modern era of biodiversity surveillance, metagenomics approaches have empowered high-throughput evaluation of compound microbial communities (Van Dijk et al. 2014).

9.6 Role of Metagenomics in Isolation of Novel Microbial Species for Disease Control

Diagnostic shotgun metagenomics offers leverage with the prospect to pinpoint heretofore unclassified microbes or efflorescent and neoteric pathogens which can be used for inducing disease resistance in plants. Benítez and Gardener (2009) utilized microbial community profiling to abstract novel bacteria strengthening soilborne plant disease suppression. They developed sequence-based terminal restriction fragment (TRF) length polymorphism (T-RFLP)-descended genetic markers to operate the testimony and seclusion of newly identified bacteria associated with damping-off pathogen repression. They isolated and classified newly evolved species, viz. *Mitsuaria* and *Burkholderia*, with steep intensities of sequence resemblance to the targeted M139 and M141 TRF. These unfamiliar isolates presented the targeted operation by abbreviating fungal development in vitro and downsizing disease acuteness in afflicted *Solanum lycopersicum* and *Glycine max* plantlets. Such studies may help in reducing the disease load in farmers' field by application of novel microbial species.

9.7 Role of Metagenomics to Address Climate Change Problems and Their Influence on Plant Vigour

Metagenomics facts and figures endure the assurance to uphold plant health management underneath changing climatic conditions. The host commune can be typecasted in labels of induced and acquired resistance levels (Garrett et al. 2006).

The environment can be defined with elaborate specification regarding abiotic physiognomy or with news about a plethora of supplementary microbial entities, such as mycorrhizal fungi; nevertheless unification amidst such datasets can be exigent (Jumpponen et al. 2010). In certain occasions, preeminent specifications regarding the dimensional and temporal constellation of these qualities can also recuperate assessments of disease threat. The role of plant disease in climate change alleviation has been acknowledged by Mahmuti et al. (2009).

9.8 Role of Metagenomics for Production of Protective Compounds for Exogenous Applications

Microbial populations relish root exudates consisting of sugars, organic acids, amino acids and salubrious secondary metabolites for community's growth and development. In retroaction, they exude treasured metabolites in the rhizosphere space that serve as energy supplies, communicators, defence units and oppressor of loathsome microbes. This assists in conceiving disease-repressing setting in the rhizosphere. Fortifying with the microbial metabolites itself might award extra advantages, especially due to exquisite control upon the production along with the pace of intensification in the root zone. Yet, counterfeiting of the in vitro microbiome for superlative yield of metabolites by microbes depicts a considerable test.

Classification of the function granted by leading microbial metabolites could conceivably release fresh routes in the region of biotic stress management in plants. Fungi and bacteria are omnipresent companion of plants. Amalgamation of metagenomics can help in deciphering the heterogeneity of lifestyles as well as their influence on the plant fitness. Endophytes have in current times acquired marked consideration owing to their envisaged promise as a resource of new and exclusive bioactive compounds to be utilized for forestry and farming goals (Rodriguez et al. 2009; Krohn et al. 2002; Ibrahim et al. 2016). Fungal endophytes have skill to safeguard their host from pathogens through generation of obstructing bioactive compounds. For instance, in *Hevea brasiliensis*, its huge multifariousness of valuable natural and endemic fungal endophytes, e.g. *Trichoderma* and *Tolypocladium*, has been testified to secure the host from pathogens (Gazis and Chaverri 2015). Likewise, in tropical trees like *Theobroma cacao* and *T. grandiflorum*, inoculation with *T. caca* endophytes dramatically interrupted the damages caused by the pathogen *Phytophthora palmivora*. In conifer trees, it was reported that subsequent inoculation of juvenile plantlets with fungal endophytes guards the host from regular toxicity by *Dothistroma septosporum*, along with reduction in consequent disease gravity (Ridout and Newcombe 2015). The manufacture of toxigenic metabolites, e.g. rugulosin, by foliar endophytes was exhibited to curtail herbivory of *Choristoneura fumiferana* (Miller et al. 2008). Needles purulent with this endophyte have considerably weakened the growth quotient of *C. fumiferana* (Miller et al. 2002; Miller 2011; Frasz et al. 2014).

By means of PhyloChip-based metagenomics, Mendes et al. (2011) revealed over 33,000 species belonging to bacterial and archaeal taxa in the root zone of *Beta*

vulgaris plants which were grown in a soil suppressive to *Rhizoctonia* damping-off disease. They exhibited that the disease-suppressive phenomenon was administered by the exudation of a thanamycin (Mendes et al. 2011; Watrous et al. 2012). Similarly, auxin phenylacetic acid, formed by gram-positive bacterial strain *Bacillus fortis* IAGS162, has a major function in persuading systemic resistance in *Solanum lycopersicum*, and it has augmented the renovation of plants' tolerance against wilt diseases triggered by *Fusarium* species (Akram et al. 2016). Actinomycetes have enormous aptitude as biocontrol agent in farming systems as they parent ionophores and enzymes comprising antimicrobial action. The utmost trivial enzymes are chitinases which can be utilized as biocontrol agents, particularly for fungal infections. *Burkholderia pyrrocinia* and *B. cepacia* are acknowledged for forming pyrrolnitrin, which shows antibacterial and tough antifungal actions (El-Banna and Winkelmann 1998; Jung et al. 2018). Metagenomics reports have also advocated that exo-polysaccharides produced by *Burkholderia gladioli* are able to evoke ISR in *Cucumis sativus* (Park et al. 2008). A sound perception of bacterial elicitor of ISR may ultimately lead to the improvement of biotic stress tolerance stratagems.

9.9 Role of Metagenomics in Plant Breeding for Disease Resistance

Root zone microbial ecology has an essential responsibility in plant development and fitness, which delivers a first line of armament opposing root diseases caused by soilborne agents. Assortment of plant genotypes must be handled in surroundings that mirror the microbe's condition in the field which are auspicious for plant-microbe interfaces. Individual pathogenic or beneficial microbes or whole microbiome profiles can be dogged to back the assortment activity in intended surroundings. Plant breeders can examine for certain root exudates which are implicated in microbiome-facilitated disease tolerance. The inheritance of stress resistance attributes can be elevated by the addition of plant's genotype, environment and microbiome communications. The recognition of genomic regions correlated with microbiome facilitated-disease repression favours designing of molecular breeding strategies. Microbiome-wide association surveys can be exploited to presume plant's vigour-related capabilities to microbial populations. Metagenomics insignia of soil microbial ecology empower plant breeders to formulate knowledgeable picks of field spots for selection and variety assessment. Chang et al. (2017) were able to tag microbial clusters correlated with yield of *Glycine max* on the basis of metagenome-wide association analysis appraising bulk soil from discrete field spots. Successively, they predicted *Glycine max* productivity based on microbiome data. Similarly, Mendes et al. (2018) scrutinized the rhythm and metabolic ability of the rhizobacterial population of various *Phaseolus vulgaris* cultivars with wavering heights of resistance against *Fusarium oxysporum* (Fox). They proposed that microbes belonging to Pseudomonadaceae, Bacillaceae, Solibacteraceae and Cytophagaceae families were more profuse in the rhizosphere of the Fox-resistant cultivar. Metagenome study showed that particular metabolic

features like protein secretion systems and biosynthesis of genes related to production of antifungal compounds, e.g. phenazines and rhamnolipids, were ample in the rhizobacterial population of the Fox-resistant cultivar.

Metagenomics-based modern acumen into the molecular basis of plant-microbiome communications bestowed scope for resistance breeding of crops (Wille et al. 2019). In the new millennium, advanced investigation of the soil microbiome and enrolment of appended tools such as metagenome-wide association studies will permit to forecast traits like disease resistance based on the root zone population constitution which can be exploited in breeding for disease-resistant varieties (Chang et al. 2017; Nogales et al. 2015).

9.10 Role of Metagenomics for Production of Disease-Resistant GM Crops

Genetic engineering (GE) counts on the point that the ideal functionality of living organisms is the practical consequence of its genetic material, more squarely the functional genes. By modifying and/or knowing and introducing an entire new gene or its portion, it will execute a distinctly changed event. The choice of target DNA fragment for GE is grounded on addition or alteration of extrinsic gene or existing gene fragment in the plant to accord a novel feature or to change the precedent one. It is simple to tap a gene accountable for the disease resistance in microbial realm than to chase modification of the genes in the plant haphazardly to pinpoint the gene, which inflates disease resistance, and reshape it to enrich its disease resistance.

The underlying approach involved in GE of plants to improve disease resistance is to report the genes that may prohibit plant pathogens or make the plant impervious to the microbial attack (van der Biezen 2001). This can be accomplished in numerous ways. The most prevalent one involves genes accountable for the production of antimicrobials, protective secondary metabolites and induction of systemic resistance (Mourgues et al. 1998; Ho et al. 2006). Metagenomics gives extensive information in a brief period about the promising genes without requiring growth/culture from the whole microbial community. With the occasional evolution of novel species, the avenues of novel antimicrobial properties possessed by them are not altogether ambiguous. Novel properties can be exploited for improvement of crop plants via genetic engineering of novel genes. Several transgenes generating compounds suitable for provoking hypersensitive reaction escorting to ISR against plant pathogens have been commissioned in genetic engineering for disease resistance (Jain et al. 2018).

The role of metagenomics in comparing GE and non-GE plants can be provided by the following example. GE of plants with *Bacillus thuringiensis* (Bt) cry proteins may incline alterations in the bacterial endophyte population connected with shoots. Therefore, Mashiane et al. (2017) have conducted metagenomics study of bacterial endophytes linked with the phyllosphere of Bt *Zea mays*. They exhibited that operational taxonomic unit (OUT) multiplicity diminished with plant growth. OTUs belonging to *Proteobacteria* were overriding in all these phyllospheres.

Also, it was found that the microbes belonging to the class *Gammaproteobacteria* were governing in Bt maize, while in non-Bt maize phyllospheres, *Alphaproteobacteria* and *Actinobacteria* were prevalent. Through hierarchical cluster analysis, they further suggested that the bacterial endophyte communities of both maize genotypes were associated differently. Further, they showed that bacterial endophyte population fluctuated more through developmental phases than among genotypes.

Metagenomics was also utilized to clear ethical concerns related to genetically modified organisms (GMPs). Subsequent to the first farming of GMPs, very momentarily queries arose related to the menace of shifting of the transgenes to soil prokaryotes with plausible ramification on the bacterial community architecture and on the outbreak of antibiotic resistance to soil and clinical strains of bacteria. For interpreting the predominance and medley of antibiotic resistance genes in soil bacteria and their wherewithal to be transported horizontally from transgenic plants to soil bacteria, metagenomics experiments were conducted by Demanèche et al. (2008). They studied the frequency and multiplicity of bla genes in soil bacteria and the consequent influence that a consecutive 10-year culture of the transgenic Bt176 maize, which has a blaTEM marker gene, might have had on the soil bacterial architecture. They proposed that soil bacteria are innately resistant to a broad spectrum of beta-lactam antibiotics partially due to the polymorphism of bla genes. Moreover, no substantial variations were witnessed in bacterial antibiotic resistance levels among Bt and non-BT maize fields. Their data was enough to deduce that the jeopardy that antibiotic-resistant genes in GMPs can pose should be premeditated as almost null.

9.11 Role of Metagenomics in Plant Disease Forecasting

Forecasting is incidental on interpreting how existing systems will counter to dimensions of feasible pressures in the future. Plant syndrome forecasting is a supervision approach operated to envision the incidence or revision in acuteness of plant diseases. Forecasting schemes are stationed on presumptions approaching the pathogen's interfaces with the host and the abiotic surrounding, i.e. the disease triangle (Agrios 2005). The aim is to precisely foresee while the three aspects, viz. host, environment and pathogen, interact in such a way that infection can arise and produce fiscal damages. Forecasting versions that provoke precise likelihoods of intricate disease systems are currently on the realm. A model of a compound disease forecasting system is the EPIdemiology, PREdiction and PREvention (EPIPRE) system established for *Triticum aestivum* that concentrated on several phytopathogens (Reinink 1986). Interesting forecasting systems may also turn out to be progressively vital with global climate change. Forecasting pathology appeals a topography dimension outlook that admits the driving bionetwork of co-operating species and the impact of heterogeneous surroundings (Holdenrieder et al. 2004). It is significant to choose distinctive biotic and abiotic influences fundamental to progressions of concern at segmental stages, to advance dense models handy for

predicting forthcoming conditions of the system (Evans et al. 2013). Prototypes of plant-microbe organizations will entail knowledge regarding genes, species dealings, physiology and activities, as well as the abiotic environment to assuredly estimate imminent episodes. Owing to the metagenomics scrutinies of genes, it is achievable to classify the operational attributes of microbial populations concomitant with peculiar environments. High-throughput sequencing-based metagenomics is an exclusive tactic for broad monitoring of disease upbringings which can be hired to produce attendance statistics and to outline the allocation of specific genotypes or phenotypes. Plus, the capability to fuse calculation of habitation distraction and quantification of phenological juncture into models via remote sensing technologies will be influential in supervising changes (Lawley et al. 2016). Metagenome annotation channels characteristically pursue to designate genes to protein function and subsequently sort them in ontology clusters to categorize diverse environments (Dinsdale et al. 2008). For instance, metagenomics information of bacteria was utilized to discriminate amid environments built on clustering of energy-transfer genes (Oberding and Gieg 2016). Viral metagenomics has also divulged the pervasive existence of chaperonins, transported by viruses among marine ecologies that were connected with archaea's thermosome proteins (Marine et al. 2017). Plant pathology forecasting models should be meticulously verified and corroborated before their employment. In the future, disease forecasting systems may turn out to be more convenient as computing potential amplifies and the volume of data that is accessible to metagenomics researchers to fabricate models upsurges.

9.12 Limitations and Challenges

Although metagenomics can be considered as swiftly emerging and reliable field for phytopathology studies, many stages of metagenomics are challenging and need to be addressed to exploit its full potential. Researchers must determine the best way to get the best sample from any environment. Low-abundance species must not be overlooked. Upgraded nucleic acid isolation protocols could assure that a metagenomics library sufficiently illustrates the whole microbial community's genome without any contamination. Spanning the studies outside the bounds of nucleic acid sequence to analyse the proteins and metabolites generated by microbial population will be strategic for interpreting how a given microbial population functions and networks with its dwelling. Lack of reference genomes, cost-effectiveness and standardization of metadata are some other aspects which need to be checked for more efficient metagenomics analyses.

9.13 Conclusion and Future Prospects

Metagenomics displays an exclusive prospect to scrutinize how microbial populations act together with crops and may ultimately accelerate towards harnessing the power of soil microbial communities to create vigorous and

disease-free crops. Discerning structure and operation of flora socialized microbial neighbourhood has wide-scale application in integrated plant disease management which is requisite for balanced cultivation. Diagnosis of the causal phytopathogen is vital for its management and control. Metagenomics is equipped with a novel exposition and powerful approach for characterizing the chief causal entities. It also helps in identification of new enzymes, antibiotics and other reagents. Metagenomics can help to explore more exotic habitats, and identification of novel gene can be utilized in crop improvement for disease resistance.

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Endophytes as Guardians of Plants Against Diseases

10

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Abstract

A huge number of different microbes are resident of interior parts of the plants which are collectively known as endosphere microbiome of the plants. There are different classes of these endophytes which are primarily bacterial and fungal organisms. Some of them are endophytic to the aboveground parts of the plants, while many are endophytic to the roots. Several biotic and abiotic factors affect the occurrence and diversity of these endophytes. These endophytic microbes have developed very vital symbiotic relationship with the plants and are involved in a variety of physiological functions. For instance, different endophytic microbes act antagonistically against several plant pathogens and are involved in the activation of plant defense responses. They act as guardians of the plants against diseases by growth promotion through nitrogen fixation, nutrient mobilization, iron sequestration, and synthesis of ACC deaminase and induction of various phytohormones. Similarly, these antagonistic microbes could directly defend the plants against pathogens through production of secondary metabolites, ROS, antimicrobial compounds, allelochemicals, and toxins. Moreover, these endophytes induce siderophore production, activate induced systemic resistance, and are involved in biosynthesis of antibiotics and proteolytic enzyme to directly

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control the plant pathogens. This chapter elaborates various classes of endophytes and mechanisms involved in the antagonism employed by different endophytes against various plant pathogens.

Keywords

Endophytes · Biotic stresses · Rhizosphere · Secondary metabolites · Plant defense · Plant pathogens

10.1 Introduction

Endophytes are microorganisms that mutually reside within host plant tissues (Stone et al. 2000). These endophytes can have beneficial effects on plant biomass and plant protection from environmental factors and pathogens. Plants have been historically allied with endophytes for about 400 million years back, which played a significant part in the evolutionary events of life (Krings et al. 2007). De Bary (1866) coined the term "endophytes" for the organisms living inside plant body (Dutta et al. 2014). Generally, endophytes include bacterial, fungal, protistic, and archaeal cells that are isolated from the surface-sterilized interior parts of the plant and considered as mutualists. Endophytes get shelter and nutrients from host plant and in return facilitate the plants for nutrient uptake and defense against stress factors (Hardoim et al. 2015). Such microorganisms have developed numerous survival strategies to live *in planta* as symbionts (Goyal et al. 2016). Moreover, endophytes have also adopted different defense mechanisms for defending themselves from various plant's physical and chemical attacks such as *Camptotheca acuminata* releases camptothecin (anticancer compound), which attacks topoisomerase I to inactivate mitotic division of cells. An endophytic fungus (*Fusarium solani*) altered the binding site of topoisomerase by certain modifications in amino acid residues to diminish the damaging effects of camptothecin (Kusari and Spiteller 2011). There are some endophytes that reside asymptomatically inside host plants, and such agents could possibly be present in almost all plant species (Saikkonen et al. 1998). In terms of association, it is possible that one endophyte species may be found in more than one plant species or more than one endophytic microbial species may be found associated with the same plant species. Although numerous endophytes may remain inactive, a number of such microorganisms have been identified to interact with pathogenic, nonpathogenic, and even endophytic species (Zabalgogeazcoa 2008).

It is well documented that land plants developed symbiotic relationship with fungi that might have helped plant evolution from an aquatic to terrestrial habitat (Plett and Martin 2015). With the passage of time, endophytes genetically adjusted themselves according to the environment of host plant (Germaine et al. 2004) and started to produce some plant metabolites (Zhang et al. 2006). They are present in different plants like herbs, shrubs, lichens, grasses, and deciduous and coniferous trees. Therefore, endophytes are very important players of the ecosystem (Sun and

Guo 2012). Endophytes may evolve from microflora of phyllosphere and rhizosphere. They enter into xylem tissues by roots due to cuts, wounds, and other cell wall openings made by certain enzymes like celluloses and pectinases (Sturz & Nowak 2000). The transmission of endophytes from one generation to another follows either vertical transmission (via seeds) or horizontal transfer (as associated other plants or soil) (Zabalgogeazcoa 2008; Herrera et al. 2016).

Current chapter is designed to explain the complete array of endophyte-plant symbiotic associations with special reference to plant pathology, biochemistry, defense mechanisms, and endophyte genetics against plant pathogens, and we have tried to summarize the endophyte taxonomy and ecological roles.

10.2 Classification of Endophytes

There are two major categories of endophytes as bacterial and fungal endophytes.

10.2.1 Bacterial Endophytes

These are nonpathogenic endosymbiotic microorganisms, which inhabit plant tissues. Novel metagenomic technologies have identified that the community of endophytic bacteria is affected by several factors including plant genetics, plant-microbe or microbe-microbe interactions, and even environmental conditions (Ali et al. 2017). Bacterial endophytes mostly belong to *Bacillus*, *Burkholderia*, *Micrococcus*, *Pantoea*, *Pseudomonas*, *Microbacterium*, and *Stenotrophomonas* genera (Santoyo et al. 2016). Bacterial endophytes are found in both above- and below-ground parts of the plants in a range from few microbes up to 9×10^9 microbes per gram of plant biomass (Chi et al. 2005). A number of bacterial metabolites (including 1-aminocyclopropane-1-carboxylate deaminase) helped in elucidating the variation in levels of ethylene. Endophytes have significant population diversity and a great potential for the biosynthesis of important bioactive compounds (Yang et al. 2018).

10.2.2 Fungal Endophytes

Based on ecological functions, host plants, evolution, and taxonomy, fungal endophytes have been further categorized into two subgroups.

10.2.2.1 Class I C-Endophytes (Clavicipitaceous Endophyte)

This group of endophytes is considered as defensive mutualists of host plants especially grasses (Clay 1988). The clavicipitaceous group of endophytes was initially identified in seeds of *Lolium arvense*, *L. temulentum*, *L. remotum*, and *L. linicolum*. Members of this group were identified to be involved in improving the ability of turfgrass to tolerate heat stress (Kane 2011). A number of

C-endophytes have now been identified, which act as plant protection agents (Varanda et al. 2016; Kernaghan et al. 2017; Leylaie and Zafari 2018). C-endophytes were found to grow better on meristematic tissues. There are three subtypes of Class I C-endophytes; in Type I endophytes, stromata (condensed somatic tissues where fruiting bodies are formed) appear on majority of tillers. In Type II fungi, stromata are produced on few tillers. It resulted in a restricted seed production and thus vertical transmission within seeds. Plants infected with Type III endophytes do not show symptoms of infection during entire period of plant development (Schardl et al. 2004).

10.2.2.2 Non-clavicipitaceous Endophytes

Members of non-clavicipitaceous endophytes (NC-endophytes) were identified from asymptomatic tissues of plants inhabiting a vast range of environments (Rodriguez et al. 2009). Based on ecological functions, *in planta* biodiversity, mechanisms of transmission between different generations of hosts, and patterns of colonizing a host, Class II, III, and IV endophytes are considered as functional subgroups of NC-endophytes (Table 10.1).

Class II endophytes belong to a fungal subkingdom *Dikarya*, which involves divisions of *Ascomycota* and *Basidiomycota*. Class II *Basidiomycota* has a few members from subdivision *Agaricomycotina* and *Pucciniomycotina* which can distinguishingly colonize roots, leaves, and even stems of the plants. Even under severe stress conditions, these fungi can form widespread colonization. These endophytes are transmitted from one generation to next through seed coats or rhizomes (Table 10.1). Members of this group can help plant in increasing biomass under unfavorable environments (Redman et al. 1999) and can even protect plants from pathogens (Samuels et al. 2000; Vu et al. 2006; Campanile et al. 2007).

Endophytes belonging to Class III are hyperdiverse endophytic fungi which are found in leaves, fruits, flowers, inner bark, and asymptomatic wood (Arnold et al. 2000; Davis et al. 2003; Kumar and Hyde 2004; Tejesvi et al. 2005; Davis and Shaw 2008). This group is involved in more multifarious and distinct ecological functions as compared to Classes I and II. In this group, the distribution of reproductive structures is mediated through herbivores, wind, or rain (Kirk et al. 2001; Selosse et al. 2008). In *Theobroma cacao*, these endophytes were involved in decreasing formation of lesions and leaf death caused by *Phytophthora* sp. (Arnold et al. 2003). However, under drought conditions, Class III endophytes negatively affected the plant growth (Arnold and Engelbrecht 2007).

Class IV endophytes are also called dark septate endophytes (DSEs). Members of this group were initially identified as brown- to black-pigmented fungus from roots of terrestrial plants (Merlin 1922). They are also called "mycelium radicis atrovirens" (MRA) or "pseudomycorrhizal" fungi because of their co-occurrence with mycorrhizal fungi. Almost 587 dark-pigmented fungi have been reported to be present in roots of at least 144 plant families (Jumpponen and Trappe 1998). The distinctive property of DSEs is their exclusive presence in roots and dark melanized functional septa. DSEs follow horizontal transmission and are ubiquitously present

Table 10.1 Endophytes, their host plants, and their PGP activities

Endophytes	Host plant	PGP activities	References
Endophytic bacteria			
Azoarcus sp. BH72	*Oryza sativa*	Nitrogen fixation	Krause et al. (2006)
Azospirillum lipoferum 4B	*Oryza sativa*, *Zea mays*, *Triticum*	Nitrogen fixation, phytohormone secretion	Wisniewski-Dyé et al. (2011)
Azospirillum sp. B510	*Oryza sativa*	Nitrogen fixation, phytohormone secretion	Kaneko et al. (2010)
Burkholderia phytofirmans PsJN	*Solanum tuberosum*, *Solanum lycopersicum*, *Zea mays*, *Hordeum vulgare*, *Allium cepa*, *Brassica napus*, *grapevine*	IAA synthesis, ACC deaminase	Weilharter et al. (2011)
Burkholderia spp. KJ006	*Oryza sativa*	ACC deaminase, nif gene cluster, antifungal action (indirect PGP)	Kwak et al. (2012)
Enterobacter cloacae ENHKU01	*Capsicum*	Unknown role in PGP	Liu et al. (2012)
Enterobacter sp. 638	*Populus*	Siderophore, IAA, acetoin and 2,3-butanediol synthesis, antifungal action (indirect PGP)	Taghavi et al. (2009)
Gluconacetobacter diazotrophicus PaI5	*Sugarcane*, *Oryza sativa*, *Coffea*, tea	Nitrogen fixation, auxin synthesis	Bertalan et al. (2009)
Klebsiella pneumoniae 342	*Zea mays*, *Triticum*	Nitrogen fixation	Fouts et al. (2008)
Pseudomonas putida W619	*Populus*	IAA synthesis, ACC deaminase	Taghavi et al. (2009)
Pseudomonas stutzeri A1501	*Oryza sativa*	Nitrogen fixation	Yan et al. (2008)
Serratia proteamaculans 568	*Glycine max*	IAA synthesis, ACC deaminase, acetoin, and 2,3-butanediol synthesis	Taghavi et al. (2009)
Stenotrophomonas maltophilia R551-3	*Populus*	ACC deaminase	Taghavi et al. (2009)
Endophytic fungi species			
Monilinia laxa	*Larix sibirica*	Unknown	Kauhanen et al. (2006)
Lophodermium piceae	*Picea abies*		Müller et al. (2001)
Lophodermium piceae *Mycosphaerella* sp.	*Picea glauca*		Stefani and Be´rube´ (2006)

(continued)

Table 10.1 (continued)

Endophytes	Host plant	PGP activities	References
Lophodermium sp. *Hormonema* sp.	*Pinus monticola*		Ganley and Newcombe (2006)
Cyclaneusma niveum *Cenangium ferruginosum*	*Pinus nigra*		Jurc et al. (2000)
Hormonema sp. *Lophodermium nitens*	*Pinus strobus*		Deckert and Peterson (2000), Deckert et al. (2002)
Penicillium sp. *Cladosporium maculicola*	*Populus tremula*		Santamaria and Diez (2005)
Ophiovalsa betulae *Trimmatostroma betulinum*	*Betula pubescens*		Barengo et al. (2000)
Phomopsis quercina *Diplodia mutila* *Dicarpella dryina*	*Quercus cerris*		Gennaro et al. (2003), Ragazzi et al. (2003)
Phomopsis quercina *Apiognomonia quercina*	*Quercus pubescens*		Ragazzi et al. (2003)
Rhodotorula pinicola	*Pinus tabulaeformis*		Zhao et al. (2002)
Valsa sordida *Trichoderma viride*	*Populus tremula*		Santamaria and Diez (2005)
Trichoderma sp. and *Bacillus* sp. *consortium*	*Triticum aestivum*		Din et al. (2018a)

in various ecosystems including harsh climates that advocated a key role of these fungi in plant ecophysiology.

10.3 Endophytic Associations with Plant

Endophytes are well known to protect their host plant from harsh environments like hot springs, stop herbivores' attack by releasing different alkaloid toxins in grasses, and also protect dicots from pests (Zhang et al. 2006). It is evident that endophytes communicate and suppress the growth of pathogens through several ways in different host plants by modulating physiology, affecting nutrient balance, producing

antibiotic and antifungal agents, or accelerating the defense mechanisms of host plant (Istifadah and McGee 2006; Zabalgogeazcoa 2008; Busby et al. 2016).

Endophytes have the ability to promote plant growth by increasing mineral uptake, suppression of plant pathogens, production of phytohormones, and biological nitrogen fixation. Colonization of fungal endophytes may protect host plants against nematodes (Schouten 2016). Similarly, it has been recently reported that endophytic bacteria like *Pseudomonas putida* and *Pantoea agglomerans* could be used to control root knot nematode *M. incognita* in different species of ornamental plants probably through HCN production (Din et al. 2018b). The endophytic diazotrophic bacteria enhance the growth of sugarcane (Cocking 2003). Another endophyte *Azospirillum lipoferum* reduces drought stress in *Zea mays* by producing gibberellins and ABA (Cohen et al. 2009). Members of *Burkholderia* sp. can alleviate ethylene levels to stimulate the growth of *V. vinifera* and *S. tuberosum* plants (Frommel et al. 1991; Barka et al. 2000). The inoculation of *Curtobacterium flaccumfaciens* protected the citrus plants against *Xylella fastidiosa* pathogen (Araújo et al. 2002). Similarly, *Arachis hypogaea* plants were also protected from *Sclerotium rolfsii* pathogen by inoculation of *Bacillus* sp. (Tonelli et al. 2011). On the basis of their living environment, the endophytes could be divided into following ecological classes.

10.3.1 Foliar Endophytes

Foliar endophytes generally exist as conspicuously localized leaf tissue infections and follow a horizontal mode of transmission. Endophytes of this group exist in almost every wild or agroecosystem (Arnold and Lutzoni 2007; Higgins et al. 2007; Rodriguez et al. 2009). Members of this group naturally colonize young leaves through hyphae or spores (Petrini 1991). Endophytes generally move into plants through leaves through wounds and stomata or even via cuticle (Herre et al. 2005). It was observed that some endophytes (e.g., family *Rhytismataceae*) can restrict predominantly to a single epidermal cell of Douglas fir leaves until leaf senescence (Stone 1987). Then, these dormant hyphae begin to colonize adjacent regions. However, according to other research works, endophytes may propagate gradually between hypodermal and epidermal cells and may inhabit intercellular regions of neighboring parenchyma cells (Johnson and Whitney 1989; Deckert et al. 2001).

10.3.2 Rhizosphere Endophytes

In rhizosphere, soil texture and soil microflora significantly influence plant growth and development (Dobbelaere et al. 2003; Basu et al. 2020; Prasad et al. 2020). This narrow soil region is rich in nutrients when compared with the rest of soil zones because it contains a variety of plant exudates, i.e., carbohydrates and amino acids that attract endophytes by providing them energy and nutrient sources (Gray and Smith 2005). A diverse array of endophytes that are present in the rhizosphere and

various plant tissues play an important part in plant growth and biomass production (Jin et al. 2014). Mostly, bacterial endophytes have been reported from the rhizosphere that proved to be beneficial for plants (Ali et al. 2017). These communities are strongly associated with abiotic and biotic elements of soil, which influence microbial persistence. It also allows enough colonization of endophytes and other microbes that modify the survival rate of the endophytes within host plant (Luo et al. 2012; Gaiero et al. 2013). These bacteria belong to a number of genera including *Acetobacter*, *Achromobacter*, *Anabaena*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Clostridium*, *Enterobacter*, *Flavobacterium*, *Frankia*, *Hydrogenophaga*, *Kluyvera*, *Microcoleus*, *Phyllobacterium*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Streptomyces*, *Vibrio*, and the well-known legume symbiont *Rhizobium* (Bashan et al. 2008). Rhizobia is a diverse and well-known group of rhizobacteria that have the ability to make a mutual relationship with legumes. They stimulate nodule development in their host plant and promote the growth of nitrogen-fixing microflora. Surprisingly, this rhizobacterial group has also been reported to promote growth of nonleguminous plants as well (Yanni et al. 1997; Lupwayi et al. 2004; Prasad et al. 2015).

10.4 Endophytes as Guardians of Plants Against Biotic Stresses

Plant-endophyte relationship offers many advantages to the host including easy nutrient access, desiccation protection, and reduced parasitism by protecting plant surface from insects and pathogens (Mishra et al. 2015). In such associations, bacteria offer a distinctive chance of biological control for plant protection against deadly phytopathogens. A number of certain endophytic bacteria have been identified that can defend a plant from soilborne fungal pathogens (Sturz & Nowak 2000). Endophytes use a number of strategies for biological control including allelochemicals, antagonism, antibiosis, competition, immunogenic response, and induced systemic resistance (ISR) (Gómez-Lama Cabanás et al. 2014; Blumenstein et al. 2015; Singh et al. 2015; Prasad et al. 2017). The ISR activity is arbitrated not only by rhizobacteria (free-living) and endophytic bacteria but also by intracellular plant growth-promoting bacteria (iPGPB). Examples include provision of ISR by *B. pumilus* SE34 against *F. oxysporum* f. sp. *pisi* on pea roots, *P. fluorescens* against *F. oxysporum* f. sp. *radicislycopersici* on tomato, and *P. fluorescens* EP1-triggered ISR in tomato and sugarcane against *Colletotrichum falcatum* and *Verticillium dahliae* (Maheshwari 2017). Thus far, comprehensive investigations are required to elucidate the mechanism of disease suppression and biocontrol (Fench et al. 2016).

10.4.1 Defense Against Herbivores

Endophytes can protect host plant from herbivores and insects by producing different toxins. A well-known instance of such associations involves *Neotyphodium* and *Epichloe* (Schardl et al. 2004). Other such species include *Acremonium strictum*,

Piriformospora indica, and species of *Stagonospora* (Ernst et al. 2003; Waller et al. 2005; Hol et al. 2007). With the advancements in genetic engineering, antipest proteins (lectins) were also studied in recombinant expression analysis (Fahey 1988). Endophytes like *Chaetomium globosum* YY-11(fungi), *B. subtilis*, and *Enterobacter* sp. express *Pinellia* ternate agglutinin (PtA) gene for biocontrol of sapsucking pests. Recombinant endophytes expressing *PtA* gene were proved bioinsecticides against sapsucking pests and white-backed plant hopper (Zhang et al. 2011).

With the application of endophytes, plants like banana, cabbage, coffee bean, ribwort plantain, soybean, and tomato have experienced a significant reduction in damage caused by herbivores (Maheshwari 2017). Endophytes have the ability to produce certain chemotoxins, which protect the host plants from herbivores such as *Neotyphodium*, *Epichloe*, *Piriformospora indica*, *Acremonium strictum*, and some *Stagonospora* endophytic species that not only promote antiherbivore defense mechanisms but also stimulate nutrient uptake, plant growth promotion, and drought tolerance to host plant species (Ernst et al. 2003; Schardl et al. 2004; Waller et al. 2005; Hol et al. 2007; Gill et al. 2016).

10.4.2 Defense Against Plant Pathogens

Pests and diseases have always been the foremost limitations for global agriculture. To avoid loss caused by pests and diseases, farmers use heavy pesticides. It is contaminating our environment and negatively affecting not only humans but animals as well. A number of compounds obtained from endophytes (flavonoids, alkaloids, steroids, and terpenoids) have been reported to have effects like anticancer compounds, antibiotics, biocontrol agents, and even immune-suppressants (Joseph and Priya 2011). The *Beauveria bassiana* fungus was found effective against borer insects in sorghum and coffee seedlings (Tefera and Vidal 2009). Similarly, *B. subtilis* acted in an antagonistic way against the fungal pathogen *Botrytis cinerea*, which causes rotting of tomatoes in storage (Wang et al. 2009). Moreover, *B. cepacia* and *Burkholderia pyrrocinia* JK-SH007 potentially act as biocontrol agent of canker disease in poplar (Ren et al. 2011). Similarly in banana, *B. thuringiensis*, *B. subtilis*, and *Bacillus amyloliquefaciens* (endophytic bacteria) protect host plant from *Colletotrichum guaranicola* and *Fusarium oxysporum* f. sp. *cubense* (fungal pathogens) (Souza et al. 2014).

10.4.3 Chemical Species Produced by Endophytes in Plant Defense

Numerous important metabolites related to plant growth promotion and protection have been characterized in the endophytic microbiome (Brader et al. 2014; Prasad et al. 2008). A variety of secondary metabolites such as azadirachtin A, azadirachtin B, camptothecin, citrinal B, cytochalasin N, diosgenin, gliotoxin, germacrane-type sesquiterpenes, ginkgolide-B, huperzine A, penicillide derivatives

and a-pyrone analogues, piperine, podophyllotoxin, and Taxol (paclitaxel) have been isolated from several endophytes. These secondary metabolites are used in pharmaceutical and agricultural industries. These endophyte-derived metabolites also play an important role in defense-related mechanisms and beneficial communication with host plants (Maheshwari 2017).

10.5 Strategies Employed by Endophytes Against Pathogens

Endophytes have gained great attention due to their capability to promote plant growth and health. They have the ability to invade and colonize the host plant tissues by using plant's organic metabolites. They have been isolated from different host plant tissues such as root zone, bark, stems, leaf blade and segments with midribs, primordia, meristem, and resin duct (Hata et al. 2002; Hata and Sone 2008; Pirttilä et al. 2008). According to literature, very limited data is reported to elucidate the molecular interaction between host plant-endophyte relationship due to their complex chemistry and environmental conditions. The study of endophytism is primarily based on plant genotype and type of endophyte. Basically, endophytes adopted two main mechanisms to affect the host plant defense system.

Endophytes can induce plant defense mechanisms against pathogen attack and other biotic and abiotic stresses by producing various reactive oxygen species (ROS), phytohormones such as indole acetic acid (IAA) and gibberellins (GA), pathogen-inhibiting volatile organic compounds (VOCs), cell wall-degrading enzymes, antibiotics, and chemotoxins (Griffin et al. 2010; Khan et al. 2012; Glick 2015; Nath et al. 2016; Kapoor et al. 2019; Kundu et al. 2020). Generally, endophytes release phytohormones to induce root growth and also generate ROS to stimulate the production of antioxidant phytoenzymes which protect the host plant from oxidative stress (Tanaka et al. 2006; White Jr and Torres 2010; Waqas et al. 2014).

It is well documented that plant-microbe interactions have ability to induce localized or systemic alterations by altering or inducing gene expression of host plant defense and metabolic pathways (Heil and Bostock 2002; Ownley et al. 2010; Mathys et al. 2012). Previous studies have shown that genes for carbon and nitrogen metabolism are expressed by colonization of endophytes with host plant (Elvira-Recuenco and Van Vuurde 2000). Endophytes release a variety of chemical signals, and host plant can detect them by chemoperception system and receptor-like kinases (RLKs), such as leucine-rich repeat-receptor-like kinases (LRR-RLKs), wall-associated kinases (WAK), lectin receptor-like kinases (LecRLKs), and Lys-motif (LysM) receptors, among others, and by plant small RNAs (sRNA) as miRNA and small interfering RNA (siRNA) (Carvalho et al. 2016) which activate a series of plant defense mechanisms by changing host plant's metabolic state (Qawasmeh et al. 2012). Recently, it has been reported that endophytic rhizobacteria and actinobacteria have capability to induce the disease tolerance in host plant by triggering certain systemic defense pathways. For instance, systemic acquired resistance (SAR) and jasmonic acid/ethylene (JA/ET) pathways in *Arabidopsis* are

expressed by infection with biotropic pathogens and necrotrophic pathogens, respectively (Durrant and Dong 2004; Conn et al. 2008). Similarly, cucumber plants induce their PR proteins and other defense-related chemical signals against *Rhizoctonia solani* KACC40111 (pathogenic fungi) when they interact with endophytic bacterium *Bacillus thuringiensis* (Seo et al. 2012). Here we have discussed different strategies used by the endophytes to combat and antagonize plant pathogens.

10.5.1 Activation of Defense-Related Genes

Plants respond to different biotic and abiotic stresses via triggering many metabolic pathways and defense-related genes which provide immunity against different pathogens such as pathogenic fungi, bacteria, viruses, nematodes, and root-feeding insects (Shine et al. 2018). The main entry site for pathogens into plant tissues is rhizospheric zone. Plant roots secrete a large number of antimicrobial compounds that protect the plant against pathogenic microorganisms (Baetz and Martinoia 2014).

Plants recognize a large number of pathogens by surface pattern recognition receptors (PRRs) and also encode different resistance (R) genes (encode cytoplasmic receptors), which activate the pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively. Several studies have shown that gibberellins (GA), abscisic acid (ABA), indole acetic acid (IAA), brassinolide (BL), and cytokinins (CK) play an important role in plant defense against pathogens (Robert-Seilaniantz et al. 2011).

10.5.2 Growth Promotion for Plant Defense

Endophyte microorganisms are capable of promoting plant growth by improving mineral uptake, aiding in plant hormone synthesis involved in growth promotion, through nitrogen fixation and by controlling phytopathogens. In growth promotion of sugarcane and other plants, *Gluconacetobacter diazotrophicus* (endophytic bacteria) has a role in N nutrition (Cocking 2003). Similarly, another endophyte *Azospirillum lipoferum* helps *Zea mays* in synthesizing phytohormones to cope drought stress (Cohen et al. 2009). *Curtobacterium flaccumfaciens* was proven effective in protecting citrus plants from *Xylella fastidiosa* (Araújo et al. 2002). Moreover, when plants of *Arachis hypogaea* were inoculated with *Bacillus* sp., a systemic resistance was induced against *Sclerotium rolfsii* (Tonelli et al. 2011). It was observed that co-inoculation of different endophytes improved nitrogen and phosphorous assimilation compared to plants inoculated with individual endophyte bacteria (Rojas et al. 2001). However, contrary results were also reported elsewhere for other endophytes (Bent and Chanway 1998).

Endophytes are very important elements of sustainable agriculture as they produce siderophores (iron-chelating molecules) to help plant in taking up solubilized phosphate and enhancing mycorrhizal growth and colonization (Wakelin et al. 2004; Das et al. 2007). Moreover, *Bacillus subtilis* HC8 (endophyte of *Heracleum*

sosnowskyi) potentially not only improved plant growth but also acted as biological control of foot and root rot diseases in tomato (Malfanova et al. 2011).

10.5.3 Defense via Secondary Metabolite Production

Numerous endophytes produce some metabolites, which can improve uptake of nutrients by plants (Fig. 10.1). It was observed that *Herbaspirillum seropedicae* (diazotrophic endophyte) can colonize grasses and can produce amphiphilic lipopeptides called serobactin (Rosconi et al. 2013). Serobactins are low molecular weight siderophores that have a very high affinity for ferric ion, and it provides iron to the cell (Schalk et al. 2011). Besides, such metabolites have also been reported that are involved in formation of biofilm, virulence, toxins, or interference with phytohormone signaling (López et al. 2008; Glick 2012; Raaijmakers and Mazzola 2012). During interactions of plants with beneficial endophytes, the synthesis and variations of phytohormones are considered very important not only for plant growth and development but also for plant stress tolerance (Hardoim et al. 2008; Glick 2012). For example, an endophyte (*Azospirillum lipoferum*) is involved in synthesis of phytohormones (ABA, gibberellins) which can help maize plant with standing drought stress (Cohen et al. 2009). Remarkably, compounds produced by these endophytes are not true phytohormones; instead they can mimic as structural analogues of natural plant hormones. For example, many plant pathogenic *Pseudomonas* species can synthesize "coronatine," which can mimic (+)-7-iso-jasmonoyl-L-isoleucine (Fonseca et al. 2009). Coronatine may function like highly active jasmonate and can result in phytotoxicity [33]. This compound can suppress defense responses and closure of stomata (Lee et al. 2013). For future research, we need to

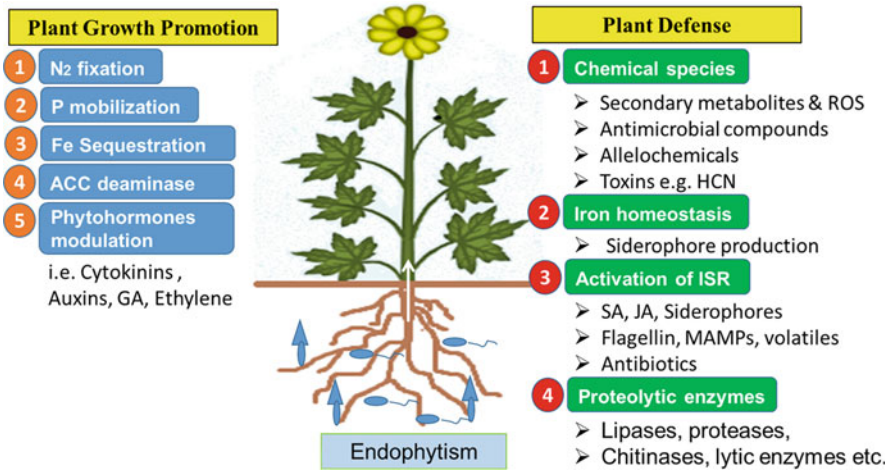

Fig. 10.1 Various strategies adapted by endophytic bacteria and fungi to promote plant growth and control pathogen growth. Majority of the endophytes enter into the endosphere through rhizosphere

see how genetic mechanisms (gene clusters including PKS and NRPS) can modulate plant hormone mimicry and which mechanisms are common among endophytes.

10.5.4 Defense Provision Through Altered Nutrients

Plants need NPK (nitrogen, phosphorus, potassium) for optimum growth and development. Generally, these nutrients are abundantly present in soil but exist as immobilized or insoluble forms of intricate compounds. It restricts accessibility of plant for these nutrients from soil. Plant beneficial microbes can solubilize these complex compounds which facilitate plant nutrient uptake. Predominantly, symbiontic microbes that can fix atmospheric nitrogen include nodule-forming actinobacteria and rhizobia. Such microbes can convert ammonia or its derived compounds into available nitrogen and represent the vital N supply for host plants under special circumstances when nitrogen is deficient in soil (Fabra et al. 2010). Numerous reports have tried to elaborate the biodiversity and dynamics of N-fixing bacteria communities (Mao et al. 2011). Some of the important endophytic diazotrophic bacteria include *Burkholderia spp., Bacillus spp., G. diazotrophicus,* and *H. seropedicae* are capable of phosphate solubilization and can promote growth and development of peanut (Taurian et al. 2009; Estrada et al. 2013). Moreover, such endophytes also enable the plants to grow on nitrogen-free medium. Additionally, phosphate-solubilizing complexes may also protect plants from pathogens in rhizosphere. Consequently, plants can grow more actively, which is important for restoring environmental pressure. A report on endophyte communities of desert-growing cardon cactus found that these endophytes were capable of solubilizing Fe/Ca phosphates to pulverize rocks (Esther et al. 2009).

There exists a significant variation in phosphate solubilization efficiency within rhizospheric populations or among diverse genera. Additionally, endophytes with capabilities of plant growth promotion via phosphate solubilization may also have other properties including production of IAA and important enzymes (Gusain et al. 2015). Acid-producing endophytes are able to enhance the solubilization of phosphatic rock (Gyaneshwar et al. 2002). Similarly, iron-chelating microbes can exert antagonistic activities by limiting iron availability to putative pathogens (Sánchez-Contreras et al. 2013).

10.6 Concluding Remarks

Certainly, plants appear to have far deep connections with endophytes, which are important for removing pollutants, inhibiting pathogens, producing fixed nitrogen, synthesizing diverse compounds, and ultimately increasing crop yields. However, most of the studies lack extensive efforts to understand and define the roles of endophytes in plants. It is only possible if comprehensive knowledge of molecular interactions among plants and endophytes can be acquired. It will be helpful in

designing manageable plant-microbe interactions with emphasis on plant disease resistance.

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Mass Production and Quality of Biological Control Agents for Pest Management

11

S. A. Dwivedi and Ajay Tomer

Abstract

Biocontrol provide quickly feasible so as to attain a well-balanced microbial association with a greatest of useful organisms prior to pathogens are present. It has long contemplated a prospective alternate to pesticidal plan of action for pest population reduction, but its impression and level of utilization worldwide remain self-deprecating and inconsistent. In this chapter discussed on the principles of biological control and recognize a series of limitation in the growth as well as comeback in conservation of natural enemies and considers the present time status. Future impact of this method embarrassment in pest management bioagents advanced toward at present time is frequently utilized for collection of external taxonomy characters for purpose of recognition and mass multiplication of various predator, parasitoids, and weed-killing agents for the purpose of reduction of pest population from agricultural crops. A succession of biocontrol techniques has been utilized on the basis "who feed on whom" query of food-web ecology; on that point of view, exotic natural enemies are introduced in India, and its conservation, colonization, and field release occurred for pest control; such successful case study with their future scope has been discussed. Bioagents improve unparalleled power to both explain and work on pest protective mechanisms inclusive of present within attraction toward attacked pests on plants. Rapid advances biocontrol will permit for evolution of still more new pest management alternative for which is comeback of to be expected restricted

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mainly by governing problem. The origin and utilization of natural enemies are well described for the growth as well as application of bioagents will need extra trials for crop production, as will the creation of new bioagents. Improvement is required in research and technology, administrative supervision, and farmer teaching to promote persistence in activity of commercial utilization of biological control for pest management.

Keywords

Biological control · Bioagents · Predators · Parasitoids · Pests · Augmentation

11.1 Introduction

Expand more successful process of agricultural pests' management by applying their bioagents, "biocontrol," analytical for three interrelated causes. Among them, first is that pests keep on causing severe harm to crops worldwide, approximately not less than $470 billion annually (Culliney 2014). Second, rapidly growing human population, integrate with get bigger per head requirements of protein, stand in a need of that loss occurred by pests and other causes are super scribe double food requirement in next incoming 10 years (Tilman et al. 2011; Godfray and Garnett 2014). Third is the present condition of pesticides are endangering due to reducing status outcome of food and effect on resistant develop in insect-pests became failure, progressively rigid on regular demands of human and environmental protection. Appropriately, agricultural boosting creation must be on the base of ecological concept in place of substantial dependence on non-autogeneration resources (Bommarco et al. 2013). In ecological point of view, escalation will magnify denotation in environment facility, like providing a bioagent that has the ability to eliminate number of pests instead of pesticides.

Narrate the historical events of the main structure of biological control to give an explanation for focus on conservation bioagents and recognized particular limitation for better application of conservation natural enemies. From many years' pest management programs biological control act as important tools in the world, it has go through rebirth in the latest 10 years that simultaneously growth of Integrated pest management (IPM) as give credit of reduction of harmful insect population. In the current chapter intentional make efforts for application of biocontrol method effective in insect-pest control it began with an outline of the general idea and protest utilization of bioagents in every normal approach of natural enemies through management. Its cursory aspect is interaction with the various elements of IPM.

Biocontrol has equatorial knowledge and advance historical events; comparatively less information is present globally in the scientific written work (Haines 1984; Altieri et al. 1989; Melo 1990; Greathead 1991; Rosen et al. 1993). Its utilization takes place at valuable place for uninterrupted and protrude cropping system helpful to uplift quickly choice of pest species that resist against chemical or plants. This condition is perfect for application of natural enemies because of great

diversification or minimum disruption of inhabitants by climatic change (Huffaker et al. 1976). Among various types of natural enemies are parasitoids; either monophagous or relatively oligophagous ones are widely utilized in pest management. Parasitoid like *Chelonus blackburni* Cameron (Braconidae: Hymenoptera) is one of the dominant and bodily stoutly exotic parasitoids accepting parasitization in few lepidopteran pests. It is an egg-larval, uniparental, and endoparasitoid introduced from Hawaii, USA, which has fairly wide host range for Lepidoptera (Raj et al. 1999). The potentials of *Chelonus blackburni* have been evaluated against few lepidopteran pests by earlier research workers from parasitization as well as efficiency point of view. The parasitoids showed parasitism in fresh eggs of *Helicoverpa armigera* laid on the leaves of pigeon pea (*Cajanus cajan*) plant (Rangadhamaiah et al. 1984). *Campoletis chlorideae* larval parasitoid is effective against this pest in chickpea (Pillai et al. 2016). Use of *C. blackburni* gave good control of cotton bollworms (Pawar and Prasad 1985). For biocontrol purpose, the large amount of parasitoid culture is required; hence mass production is an important aspect in biological control program.

11.2 Approach of Biological Control

Bioagents have been applied for reduction of pest population in present condition. Still, from the past 100 years, a considerable rise in the utilization as well as our apprehension of their working nature is observed for it became beneficial in pest control. The latest forward in systematics molecular makes redundant focus on classification of beneficial insects order Hymenoptera in different groups (Sharkey 2007), quickly spreading this information worldwide with the help of Internet sources. Recent advances study of beneficial organism behavior for searching host (Smid et al. 2007; van Nouhuys and Kaartinen 2008) and generative anthropology are disclose astonish difficulties occurred in bioagents life that problems create prospective recent develop methods for its influence of own development. Without being affected by great experience in the application of bioagents, it was up to 1919 that the term biological control is evidently utilized first time by the late Harry Smith at the University of California (Smith 1919); its discussion occurred on the scope, importance of advance tools in pest management, and its definition (Nordlund 1996) "Study of importation, augmentation, and conservation of beneficial organisms to regulate population densities of other organisms." Definition of biological control is given by DeBach (1964).

In other words, he defined biological control in such way as the destruction or suppression of undesirable insects, other animal pests, and plants by introducing incensement or artificial rearing of their natural enemies. Its efforts carried with natural enemy up to this time can be well ordered in three normal approaches like importation, augmentation, and conservation for bioagents (DeBach 1964).

11.3 Principles as Well as Procedure of Biological Control

The principles and rearing methods of bioagents include four steps, i.e., introduction of natural enemies, colonization, augmentation, and conservation.

11.3.1 Introduction of Bioagents

Importation of natural enemies is generally called "classical biological control" which shows past prevalence applicable to bioagents. It normally includes establishment of bioagents for pests that have migrated in fresh new locality. The way of releasing bioagents for a target pest success cases is studied carefully to find fault with its side effects on nontarget, Bioagents should be imported from similar place having same environmental condition free from alternate host and other biological competitors for host. Bioagents have physiological suitability for its host and have developed adaptation ability of its host plant ecosystem with low dispersal rate; these points consider for successful introduction of natural enemies.

11.3.1.1 Some Successful Examples

In the duration starting from 1890 to 1960, nearly about 2300 natural enemy species are inaugurated, roughly 600 globally in different localities for controlling pests**.** After complete establishment of such bioagents counted to be 34%, total management of decided pests has 16% conditions, and another some stage pest reduction attains extra 42% (Hall and Ehler 1979; Hall et al. 1980).

11.3.1.2 Examples of Successful Biological Control in India

Apple woolly aphids *Eriosoma lanigerum* in Assam are controlled by using parasitoid *Aphelinus mali* from England (1920). *Icerya purchasi* of wattle tree at Nilgiris and Kodaikanal are controlled by using predators vedalia beetles *Rodolia cardinalis* from the USA (1931). Control of apple San Jose scale *Quadraspidiotus perniciosus* using parasitoid *Prospatella perniciosi* from China (1958–1960), castor semilooper *Achaea janata* by using *Telenomus* spp. from New Guinea (1964), rhinoceros beetle *Oryctes rhinoceros* utilizing predator *Platymeris laevicollis* from Zanzibar in coconut plant (1965) *Cotesia vestalis* is an important parasitoid, mass multiplied, and used against the diamondback moth *Plutella xylostella* globally (Saini et al. 2019).

11.3.1.3 Pest Resistance Against Bioagents

Generally, in the use of biocontrol for the management of target pest for more than 100 years, there is only single target pest created resistant against its natural enemies. Larch sawfly, *Pristiphora erichsonii* (Hartig) (Hymenoptera: Tenthredinidae), modified its protection capability against the parasitoid *Mesoleius tenthredinis* (Morley) (Ichneumonidae: Hymenoptera), when these bioagents get entry in Canada for the management target host (Messenger and van den Bosch 1971; Pschorn-Walcher 1977). It indicates importation bioagents are most feasible method of pest reduction.

11.3.2 Colonization of Natural Enemies

It is mechanism for manipulating an organism to establish it in new locality called as colonization. It is most critical operation in biological control and to make it successful site selected for multiplication completely protected from pesticide residues and cultural practices. It should be kept in low temperature to restrict its movement. Adult stage is in case of parasitoid, and predators are considered to be appropriate than the immature stage because adult can easily cope up with new climatic condition and is less affected by environmental stress. It should be released in field at the vicinity of suitable host at the time of transportation, container must be with suitable relative humidity field, and release takes place in morning and evening time in order to avoid direct sunlight before mating and feeding on food material are completed. On the base of host, searching ability depends on the number of natural enemies' release. Regaining chance is undertaken quickly after starting release to be certain if released natural enemies remain alive and control is being provided.

11.3.2.1 Assessment of Natural Enemies

For assessing the releasing results, minimum of 3-year time duration given after that effectiveness of natural enemies should take place on parameter like comparing host number of the pest prior to and subsequent to release, correlating population change of natural enemy and host, and estimating the population on plots in the absence of natural enemy. There are two important systems that are normally followed for judging its success:

A. Experimental procedure (exclusion method) – this method can be achieved by handpicking, chemical barrier of insecticides, and use of ants.
B. Analytical procedure (life table analysis method) – in this method, life and mortality factors are determined in the light of various environmental factors for various stages. Data is analyzed to correlate between pest density and change in density as well as the amount of mortality resulting from each counted mortality factors.

11.3.3 Augmentation

Cover all activities planned for enlargement the result of existing bioagents and its mass multiplication occurred through culture, release occurring at interval (inoculants or inundate) establish a colony to reduce domestic or exotic pests. It is broadly identified by common people in a short time of US main purpose of extensive presence of bioagents like lady beetles, *Hippodamia convergens* Guerin-Meneville, and praying mantis from garden and nurseries (Cranshaw et al. 1996).

11.3.3.1 Scientific Base for Augmentation

It has been latest find fault with (Collier and van Steenwyk 2004) as well as discussed (van Lenteren 2006; Collier and van Steenwyk 2006). Writing materials

for scientific base, successes, advantageous of natural enemy apply for pest control. Few problems regarding natural enemies have been talked over in the back. There are few scientists' opinions for evolution of anticipating models for the help in application of enhancement bioagents (Huffaker et al. 1977; Stinner 1977; King et al. 1985; Van Lenteren and Woets 1988; Ehler 1990), yet for many times, it fails (Parrella et al. 1992). Reason behind deficiency of assist information of many boost up approach suggest could not create at the point of rates, way of implementation that give to be expected outcomes. Improper releases of bioagents with incorrect dose given result in failure of pest management and give fluctuation in enhancement of natural enemy (Hoy et al. 1991). Still great long steps have been taken for the upgrade of the condition (van Lenteren 2003); lots of clarifications have been given behind for deficiency of research work to contribute to the augmentation. Surely enormous organization problems elaborate in manage the extensive statistic properly comprehensive works necessary to successful judge bioagents accretion (Luck et al. 1988). Next feasibility consider on resemblance in connecting enlargement of allow to leave and the chemical pattern has demoralized research engrossment in biological control (Parrella et al. 1992). In this point of view, augmentation to look at the least sustainable among three types of principle biocontrol, reason behind be in need of go on with external load.

11.3.4 Conservation of Bioagents

It is noticed that humans have an effect on resident natural bioagents in a crop ecosystem and then operate those determined to increase the ability of natural enemies for pest population reduction. DeBach (1964) considered conservation of bioagents in the environmental change to provide protection and intensify its activity. Such work scope of from proper way use of pesticide manipulate in bioagents niche in crop (Barbosa 1998; Pimentel 2008). Conservation of bioagents can be achieved by the following.

11.3.4.1 Rationalized Use of Pesticides

Apparently general method regarding pest population management that shows bad effect on natural enemy in the crop its result, for conservation of bioagents proper dose and way of application of pesticides those have fewer residual effects has contemplated as valuable tools in IPM (Stern et al. 1959; DeBach 1964; Newsom and Brazzel 1968; Mishra et al. 2018). Application of chemical occurs in such ways that became beneficial for natural enemies including treatment done only when pest population crosses the economic threshold level, use of only safer element and less toxic formulation selected for bioagents, application of low-price effective chemical having short residual effect, and temporal and spatial separation of bioagents as well as chemical (Hull and Beers 1985; Poehling 1989; Ruberson et al. 1998). It is concluded that use of chemical for reduction of pest population in integrated pest management programs is commonly on the base of sampling and survey pest populations to decide position of ETL or not (Pedigo 1989) despite the fact that

some work has been completed for the incorporation of beneficial insect sampling to make decision before use of chemical. Pesticides affect natural enemies by repelling and reducing the longevity, fecundity, and searching ability as well as decimating the population of flying insect that can be avoided by use of nonpersistent chemical; avoid row treatment by it and use minimum and recommended dose.

11.3.4.2 Provide Food and Shelter

Bioagent mass multiplication takes place in unsprayed chemical habitat; hence parasitoid could be released in such place to create reservoirs. Provide supplementary food material for adult parasitoids like pollen, and nectar-bearing flowering plants are planted on the bund and nesting boxes and bird perches that act as suitable place to encourage buildup of predators.

11.3.4.3 Effective Management Practices

This method covers management of chemical residues from soil, water, and crop by change in cropping patterns and provides alternate resources for bioagents (Barbosa 1998; Pimentel 2008). Generally, the main aim of this approach is to increase density of resident number of bioagents or its mass multiplication to increase their benefit in pest control. Ehler (1990) highlighted that several management techniques developed for conservation of natural enemies have been of scholastic quite than empirical engrossment which is not extensively applied in IPM model, and appreciable quantity of investigation has been carried on in that area newly and has arrived to be considerable prospects in succeeding use in integrated pest management (Jonsson et al. 2008). One feasible clarification for less chance of success in implication bioagents contrast in the rates establish of newly release natural bioagents are deficiency of available resources for bioagent in crop ecosystems. Delivery of such resources to conserve natural enemies' techniques advice for single way of upgrade the achievement for both augmentation and importation in advanced toward united biocontrol (Gurr and Wratten 1999). Chisel ploughing is helpful to protect beneficial insect for soil-inhibiting pests; sufficient amount of research has been directed in the direction of comprehension impact of decreasing tillage practices on beneficial insects. In few cases, conserved tillage work has manifest to raise the number of bioagents (Gaylor et al. 1984; McCutcheon et al. 1995; McCutcheon 2000; Tillman et al. 2004); in that period, others are not affected to decrease (Ruberson et al. 1997; Gencsoylu and Yalcin 2004). A plenty of work done on soil-living insect bioagent has concentrated on carabid beetles, *Calleida decora* (Fabricius) (Carabidae: Coleoptera), remarkable general predator which grows up in agricultural field annually (Thiele 1977; Kromp 1999; Menalled et al. 2007). Tilt practices influence population of carabid beetle directly death from this operation, or indirectly loss of prey resources as well as affect niche from start to end (Hance et al. 1990; Thorbek and Bilde 2004; Shearin et al. 2007) reported on insect feeder carabid beetles are more sensitized in soil intercultivation operation than plant consumer beetles. Due diversification and very large quantity of carabid incidence recommended for decrease tillage, it is example of insect feeder beetle is significant more a plentiful in standard

tilt operation (Carcamo 1995; Menalled et al. 2007). Change in relative humidity increases action of fungal pathogens, by utilize organic manure protect predators and soilborne microbes antagonists' of insect-pest. Kept stubbles and heap of weeds to maintain predator number reduction of ants or their physical exclusion increase successes of predators.

11.3.4.4 Impact of Plant Types on Bioagents

Leaf pubescence affects searching efficiency, oviposition, and prey consumption. Color of leaf, flower, and fruit play a key role in the attraction and repelling of parasitoids and provide good shelter for entomopathogens. Morphological characters of the host plant and differences in profile of volatile contents between varieties increase effectiveness of natural enemy. Trap crops are also helpful to increase overall effect of parasitoid and several predators (Tillman 2006). Parasitism of mustard (*B. napus* L.) pest *Psylliodes chrysocephala* (Chrysomelidae: Coleoptera) affected by the ichneumon wasp *Tersilochus obscurator* Aub is unaffected due to chemical treated in a border trap crop like turnip rape *B. rapa* L. (Barari et al. 2005). Comparison of predators and parasitoid mentioned on various characters is given in Table 11.1.

Table 11.1 Comparative study of between predator and parasitoids

Characters	Predator	Parasitoid
Definition	A free-living organism throughout its life, kills its prey, is usually larger than prey, and required more than one prey to complete its development	It is insect parasite of an arthropod which is parasitic in immature stage adults which are free-living
Term coined by		O.M. Reuter (1913)
Size and capacity	Large and stronger	Small and weak
Intelligent	More than prey	Not more than host
Habitat	Independent	Dependent
Life cycle	Longer	Short
Destruction of pest	Prey is seized, killed eaten immediately	Take long time to kill host
Killing capacity	Several preys kill in short period to obtain food	Required only host and provides food for offspring
Body organs	Mouth parts and locomotory organ well developed	Not so well developed
Food habits	Not specialized	Specialized

11.4 Mass Production Techniques of Effective Parasitoid

11.4.1 Mass-Rearing Procedure of Egg Parasitoid, *Trichogramma* Species

Trichogramma species commonly called as wasp is an egg parasitoid of many lepidopteran insects-pests. Its rearing occurred on the embryonic stage of rice meal moth, *Corcyra cephalonica. Corcyra* moth fresh eggs are exposed in UV light for sterilization (15 W for 30 min) for causing mortality of embryonic stage. These eggs are stuck on trichocard (30 × 20 cm) divided in 30 rectangles (7 × 2 cm); drawn lines contain uniformly fine layer of gum at the rate 6 cc on each card. 1 cc eggs will contain approximately 18,000–20,000 eggs. Card inserted into large polythene bags (45 × 30 cm) containing nucleus eggs card at the ratio of 1:6 to fresh eggs and exposed for 2 days. The adult parasitoid of *Trichogramma* species shown in Fig. 11.1 feeds with honeycomb applied in inner sides of the tube that attach compact with mark in cloth with elastic bands. Card changed subsequencing to 1 day, replaced with new fresh card. It is changed for 3–4 days till the survivability of female is capable for egg deposition. Female lays its egg mass on its host eggs, which turn black after 3 days of parasitization. At this stage, the parasitized eggs allow to release in field for fortnight interval kept in 10 °C. The parasitoids appear a week after parasitization under normal temperature. When kept in cold condition, the cards are taken out and kept at normal condition for a day before allowing leaving in field card cut into smaller strips at proper lines and stapled lower side of leaf.

Successful examples are 7–9 release of *T. chilonis* and *T. japonicum* at 1 lakh/ha starting at 30 days after transplanting for paddy stem borer, *Scirpophaga incertulas*, and rice leaf folder, *Cnaphalocrocis medinalis* and *Plutella xylostella* (Navik et al. 2019). Weekly release of *T. chilonis* at 1,25,000/ha from 4- to 11-week stage of sugarcane crop for internode borer and same species that allow to leave in cotton at 1,50,000/ha against bollworm parasitism is high in the fields of more eggs per card

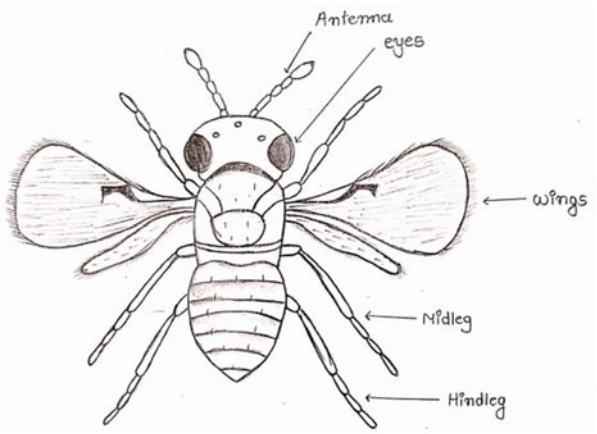

Fig. 11.1 Adult of *Trichogramma* species

throughout the trial as compared to less number of egg inoculation. It is observed that emamectin benzoate is a safer insecticide for *Trichogramma* (Qasim et al. 2018).

Develop resistant against chemical: endosulfan resistant strain developed by NBAIR formally PDBC, Karnataka, and release for private industry for marketing with commercial name endograma. This strain additionally modified forbearance against monocrotophos and fenvalerate. It is generally at the place, where use of *T. chilonis* and spraying of mention chemical for the management of pests (Jalali et al. 2006).

11.4.2 Mass-Rearing Procedure of Larval Parasitoids, *Bracon hebetor* and *B. brevicornis*

Bracon species is an external gregarious larval parasitoid, fully grown caterpillar of *Corcyra cephalonica* used as food material for the purpose of mass rearing. The bigger end of chimney is wrapped by muslin cloth. *Corcyra* larvae are placed on the muslin cloth and tightly wrapped with another cloth tied up with elastic band. Adult parasitoids soon after emergence are held in glass jar and feed with raisins after mating; two adult females of *Bracon* are released to every one rice meal moth caterpillar though small side of chimney that is wrapped with one more cloth. Female *Bracon* lay 8–12 eggs on the ventral side of caterpillars, and eggs hatched within 28–30 h. Larval and pupal periods are 3–4 and 2–3 days, respectively. Parasitized larvae separated after 1 day slowly with the support of forceps on paper plates keep away from drop down of the deposited on the external part of host body. Parasitized larvae are left undisrupted up to pupal stage after that remnant of the deadly caterpillar detached. Pupa is kept in glass jar for emergence of adult of *Bracon hebetor* given in Fig. 11.2 and adult feed with raisins life within 7–9 days completed *Bracon hebetor* is allow to leave at eight thousand adults/ac against bollworm of cotton crop (Mahdavi et al. 2013) and *B. brevicornis* at 10 adults/tree for coconut pest (BHC) *Opisina arenosella.*

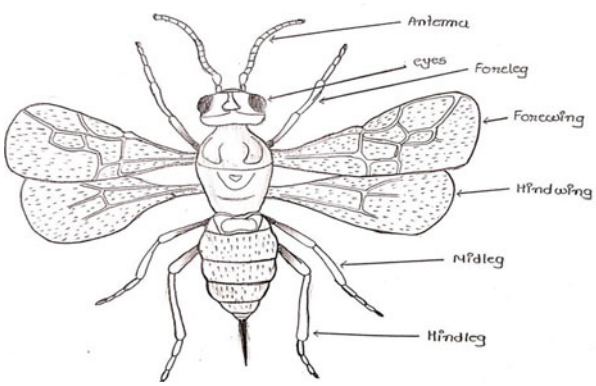

Fig. 11.2 Adult of *Bracon hebetor*

11.4.3 Mass-Rearing Procedure of Larval Parasitoids, *Chelonus blackburni*

It is egg and larval parasitoid; its mass multiplication occurred on a set of 100, and fresh egg mass of *Corcyra* (not exposed to UV) is stuck on card having 5 × 5 cm in size. Card having eggs is exposed for newly emerged parasitoid *C. blackburni* adults in plastic container at 100:1 for the purpose of avoiding super parasitism. Windows with plastic mesh for air passing in vessel present. First cotton swab absorbed in 10% honey formulation provided as food for adult and second absorb with drinking water are also kept inner side from opening which is come to end tightly with a cloth wrap by cotton plug. In succeeding 1 day disclosed to *C. blackburni*, adult and parasitoid eggs are transferred into another jar having 500 gm sterilized cumbu medium. Within 1 month, the parasitoid developed inside rice meal moth larvae and spin small white cocoon adjacent to carcass of consumed host, and finally adults appear from the pupa prepared in the rearing cumbu mixture after finishing growth on meal moth caterpillar. Adult of larval parasitoid, *Chelonus* species, given in Fig. 11.3 every day emerged adult collected by aspirator. Longevity of adult is 25 days, within that duration having 400-egg capacity. These natural enemies also multiplicities on lepidopteran pest potato tuber moth (PTM): *Phthorimaea operculella.* Group of 1500 eggs deposited on stapled to a card and wrapping cloth.

It having near about 1 day after laid eggs is disclosed to *C. blackburni* adults in vessel having size (14 cm × 11 cm) is converted into *C. blackburni* growth chamber by prepared windows and plastic mesh attach for air passing purpose. Among two cotton swabs, the first one is dipped in 50% honey-mixed water, and second one in water is also kept inner side from opening which is shut down tightly with the help of plug. Egg card of this adult gets disclosed to *C. blackburni* for 1 day and transferred on potatoes having small hole that give extra arrival place for this pest caterpillar for

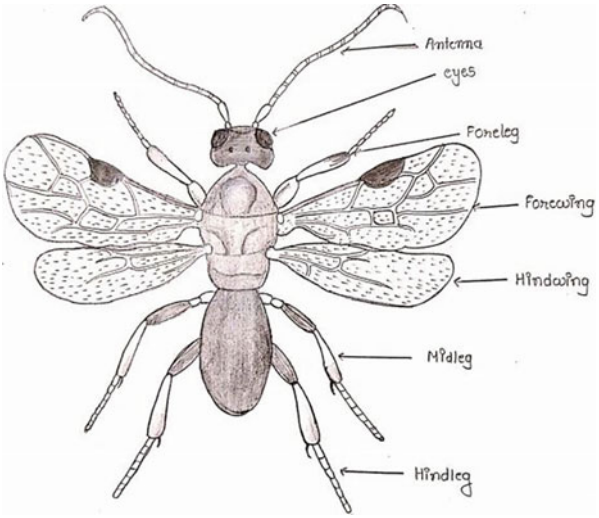

Fig. 11.3 Adult of larval parasitoid, *Chelonus* species

exposure to *C. blackburni* kept in same container. Bottom end of vessel is filled with sand after sterilization. In the duration of 25–27 days, adults start appearing into the sand pupae formed in sand or sometimes inside from infested potatoes after completing development of this pest in potatoes. Longevity of adult 23–31 days and their fecundity is about 288–390. 1:50 ratio of parasitoid with host should be maintained and every day freshly deposited eggs collected with fine brush. Appear parasitoids are collected daily and transfer into cotton field at the rate of one per plant or eight thousand per acre. It is effective against lepidopteran pest of vegetable crops (Halder et al. 2018).

11.4.4 Mass-Rearing Procedure of Pupal Parasitoids, *Tetrastichus israeli* and *Trichospilus pupivora*

These are pupal parasitoid can reared on pupa of coconut (BHC) *Opisina arenosella*, chickpea pod borer, tobacco caterpillar, *Euproctis lunata* and *Ergolis merione*, etc. fresh pupa of this host insect are transferred at the rate of 5/tube size of 15 × 2.5 cm. Adult food purpose every tube fill up 50% honey solution and transfer 30 female after mating in tube. Adult of pupal parasitoid *Trichospilus pupivora* is shown in Fig. 11.4. The newly appeared adult parasitoid consumed small drop of honey on a wax containing paper kept in vials. Newly converted pupae are provided for parasitization to the parasitoids. In succeeding 2 days, parasitized pupae are shifted in the test tubes for arrival of parasitoid, having gregarious natures which finish life stages in pupa of the target pests. Immature as well as resting stage completed within 6 and 8–10 days, respectively; field transfer occurred at the rate of 20 adults/tree for coconut (BHC), *Brachymeria nephantidis* (Chalcididae), or *Tetrastichus israeli* or *Trichospilus pupivora* (Nor Ahya et al. 2019).

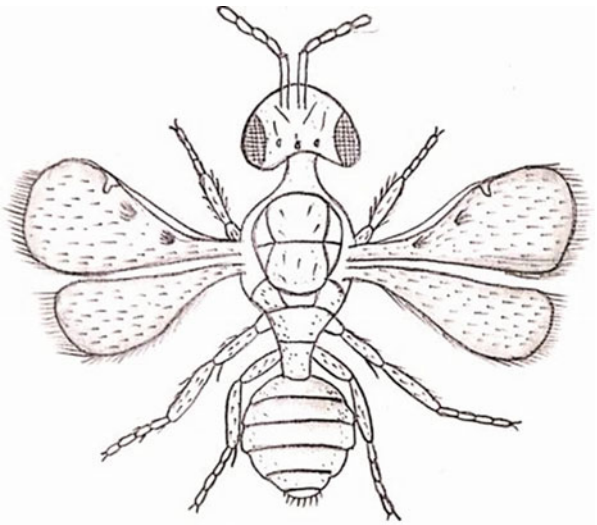

Fig. 11.4 Adult of pupal parasitoid *Trichospilus pupivora*

11.5 Mass-Rearing Procedure of Effective Predators

11.5.1 Mass Rearing of Ladybird Beetle, *Coccinella septempunctata*

Adult of ladybird beetle, *Coccinella septempunctata*, shown in Fig. 11.5 feeds on soft-bodied insect mealybugs, aphids five pair of predators released on ten medium-sized potatoes well infected with aphids, placed in glass jar, eggs deposited on potato tubers are peel off gently with the help of sharp blade and left for further development. The eggs about to hatch are placed in tube along with aphid as prey to prevent cannibalism and reared undisturbed for 2–3 days (Jazem et al. 2013). Ladybird beetle deposited their egg masses in bunch, at the sex ratio 1:1 female laying in range of 1–25. Female and male predator survived ranged from 21 to 26 days and 24 to 29 days, respectively. In incubation over from 2 to 3 days, grub stages pass in four instars. Aphids' consumption by every instar grub is 35, 63, 96, and 290, respectively, in lab condition. Developmental period of the predator completed from 16 to 21 days in the conditions of 23 ± 2 °C temperature and 60 ± 5% relative humidity.

It is noticed that parasite incidence occurred at larval as well as pupal condition, i.e., the braconid wasp, *Perilitus coccinellae* (Schrank). Before attaining pupal stage, final instar grub stops consumption, no food needed to larvae. Shifted on carton plats in cages for pupation. Adult beetles are blazing colored, that is, the deterrent colors that inform the predators are toxic. If interrupted, some species have ability to release a power full smelling liquid in yellow color to discouragement to host in opposition him. Freshly becoming visible adults are bright yellow without marks; later the markings start to appear. The color reddens enough slowly. *C. septempunctata* L: the egg color change into gray before breaking of egg coat that completed within 5 min after egg coat damage the corn look as a translucent white vacant carapace. After breaking of embryonic stage, newly emerged grub stays in folk for period of 1 h and then crawls out to search about food (Sarwar and Saqib 2010). *Coccinella*

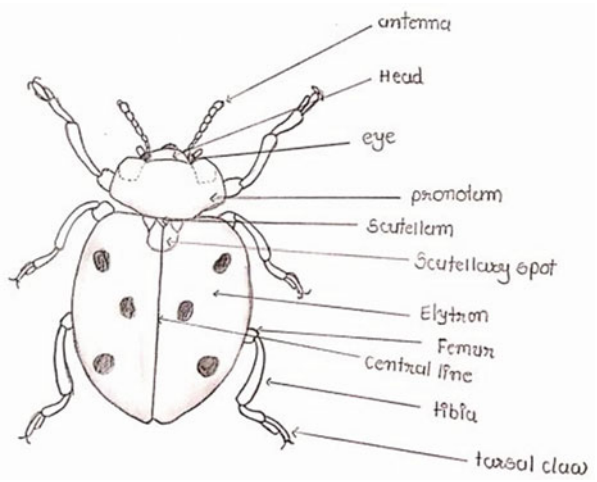

Fig. 11.5 Ladybird beetle *Coccinella septempunctata*

septempunctata and syrphid fly like predators are helpful for mustard aphid *Lipaphis erysimi* population reduction (Dwivedi et al. 2018).

11.5.2 Mass Rearing of *Cryptolaemus Montrouzieri* Mulsant

It is a promising predator for suppression of mealybug (Chacko et al. 1978; Mani et al. 1990; Singh 1996), scale insect, and aphids. Pumpkin is utilized for rearing of grapevine mealybug in the laboratory Ripened pumpkin is taken and outer surface is sterilized by 0.1% Dithane M-45 and dehydrated. Transfer of the newborn crawlers' nymph of this pest above fruit for increase population on external surface of fruit in a dark room. Completely infested squash fruit with pest is kept in (30 × 30 × 30 cm) size cage wrap by cloth from all side glass door at front side present. Uncover the squash of adult for egg deposition; separate this subsequently for 2 days. Hatched grubs feed and develop on mealybugs. Full-grown grub pupation occurred in folded paper present at the bottom of caged and in the point of view adult emergence collect pupae keep in another cage. Adult of *Cryptolaemus Montrouzieri* is given in Fig. 11.6. Development and rearing of this predator take place on freeze-dried artificial diet in lab condition (Venkatesan et al. 2001) for citrus and grapevine mealybug release 10 beetles/tree; before the release of predators, the ant movement should be arrested.

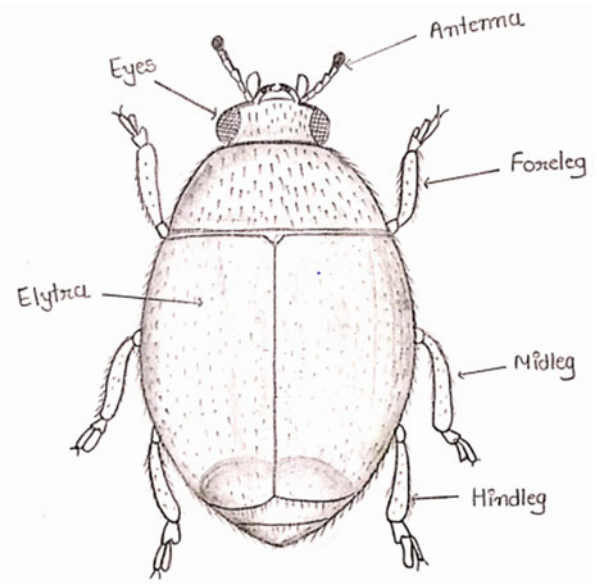

Fig. 11.6 Adult of *Cryptolaemus montrouzieri*

11.5.3 Mass Rearing of Green Lacewing, *Chrysoperla carnea* (Stephens)

Aphidlion, *Chrysoperla carnea* (Stephens), belongs to order Neuroptera and family Chrysopidae which is an international predator; its incidence is found in different crop ecosystems extensively. Preliminarily, many researchers like Henry (1979, 1985, 1993), Bram and Bickley (1963), and Brooks et al. (1994) have analyzed its work in the reduction of agricultural pests. Predator assessment may be up to 75% prosperous by biocontrol in pest management which is credited to the entry of these natural enemies and allows to live in field for consumption of pest (Williamson and Smith 1994); immature stage consumes on lots of food and effective biocontrol agents for reduction population of various plant damaging insects-pests (Mcewen et al. 2001). Single larval stage has able to eat quickly nearly about 500-aphid population in its larval period and no any question mark that perform of key role as effective bioagents against of many small size, soft-bodied sap feeder pests (Michaud 2001). Biology of this natural enemy completed within 25, 31, and 45 days on hosts *A. craccivora*, *A. gossypii*, and *C. cephalonica*, respectively. A single larva of *C. carnea* feeds on *A. gossypii* and 97.33 eggs of *Corcyra cephalonica* followed by *A. gossypii* (80.00 ± 2.65 nymphs/adults) and *A. craccivora* (64.33 ± 0.67 nymphs/adults) per day (Akshay Kumar et al. 2019). It is generally known as aphidlion because it acts as effective predator against aphid homopterous pest at larval and adult stage. Its rearing completed within two steps, viz., larval and adult rearing.

Larval rearing: Larvae generally feed on frozen eggs of *Corcyra* moths. In freshly emerged immature stage, rearing starts in separate vials or in cage having hexagonal structure of cells. In nearly about 16,000 to 18,000 eggs, *Corcyra* eggs are necessary for rearing of 5–6 grubs of natural enemies. Every cell of grub along with enough quantity prey is kept followed by wrapping with white paper for further development and pupation. After a week, cocoons gathered and are placed in different jars for appearance of adult. Freshly appeared adults are kept in caged jars with 20% solution of cotton swab and pollen grain of caster as food material for adult. Jars are protected by black wet cloths, since these prefer for deposition of eggs on black cloth. The stalked eggs are deposited on black sheet. The embryonic stage gets destalked with brushing having sponge piece. The eggs sheet can be stored at 10 °C for 21 days. On the 3rd day, eggs turn into brown and are ready to hatch.

Adult rearing: Mature stage shifts into pneumatic glass trough or G.I. round feeding container having size of (30 × 12 cm) covered by brown color sheet which acts as egg-laying site. More than 250 numbers of adults are shown in Fig. 11.7 (approximately 60% female) permitted into every container and protected by whitish nylon cloth. Three places with three moist foam sponges and protein-containing food material are placed for its consumption. Deposited stalked eggs on the brownish sheet are stored at 10 °C up to 21 days. Need base transfer eggs mass in field; the egg deposited sheet is place at normal condition and changing of eggs color brown and hatching occurred on the 3rd day.

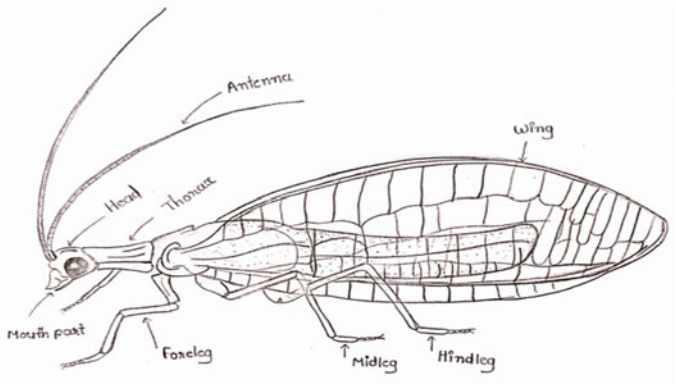

Fig. 11.7 Aphidlion, *Chrysoperla carnea*

Table 11.2 Successful predators in biological control imported from other country

Natural enemies	Target pest	Imported country	Year	Crop
Rodolia cardinalis Mulsant (Coleoptera: Coccinellidae)	Cottony cushion scale, *Icerya purchasi* Maskell (Homoptera: Margarodidae)	USA	1926	Early black wattle
Platymeris laevicollis Distant, (Hemiptera: Reduviidae)	*Oryctes rhinoceros* (Linnaeus) (Coleoptera: Dynastidae)	Tanzania	1965 (Sankaran 1974)	Coconut
Curinus coeruleus Mulsant Coleoptera: Coccinellidae)	Subabul psyllid *Heteropsylla cabana* Crawford (Hemiptera: Psyllidae)	Thailand	1988 (Jalali and Singh 1989)	Subabul
Cryptolaemus montrouzieri (Coleoptera: Coccinellidae)	Mealybug (*Planococcus citri*, *P. lilacinus*, *Ferrisia virgata*, *Maconellicoccus hirsutus*)	Australia	1898	Fruit crops
Amblyseius chilenensis Phytoseiulus persimilis (Acari: Phytoseiidae)	Spider mites, *Tetranychus* spp.	USA UK	1984 (Singh 1994)	Bean, okra, and strawberry
Euglandina rosea (Stylommatophora: Oleacinidae)	Giant African snail, *Achatina fulica* (Pulmonata: Achatinidae)	Bermuda	1950	Ornamental, vegetable, and pulses

On the 2nd day, eggs convert in to destalk with the help of brush with a sponge piece. First instar grubs are selected for culture (or) field transfer. For pest control, some predators imported from other countries successful predator list given in Table 11.2.

Rearing of adult also takes place on different artificial diets in lab condition (Sumera et al. 2016). The freshly hatched grub of this predator is released in cotton fields at the rate of 20,000 to 40,000/acre for three to five times at 10-day timespan for the management soft-bodied insect pests.

11.6 Classical Biological Control of Weeds

The main specialty of weeds is their unwanted appearance, unpleasant characters, and ability to be modified for disturbed environment measures adopted for its management; through it, there is approximately 11.5% annual decrease of food production in the world (Combellack 1992). Biological control for weed management involves application of insects to attack on harmful plant to place at or below sensible stage in the absence of significantly have an effect of usefulness and wanted plants. It is clearly demonstrated that biocontrol process does best on extensive attack by a single weed plant, which normally happens in land suitable for grazing livestock and crop field or in aquatic habitat. Despite much good favorable outcomes of classical biological weed management in waste as well as fallow land or large aquatic condition, it is undeveloped to the point that it has any significant effect on reduction of weeds in agricultural crops. Biological control involves approaches like inoculative and inundative for weed population reduction.

Bioagents like insects, nematodes, pathogens, animals, fish, birds, and their toxic products are excellent weed killers. Singh (2004) reported that in India, highest favorable outcomes with bioagents were obtained in biological control of water weed (55.5%), Homoptera pests in crops (46.7%) followed by land weeds (23.8%). McFadyen (2000) lists out 44 weeds, which were able to be effectively managed in the world using release insects and pathogens.

11.6.1 Advisable Characters of Weed Killer Insect

It must effective in destruction and management of weeds, it is preferably bore in to plant act inside of plant feeder in unwanted plants leaf consumer have also set up same successful by check of weeds. It has able to increase population without affecting other parasitoid and predator. In the lack of weed plant, it should not transfer on crop to convert as pest; list of effective weed killer insect is given in Table 11.3.

Table 11.3 Effective weed killer insect along with target weed plant

Bioagents	Target weed	Origin	Year	Reference
Cochineal scale insect, *Dactylopius opuntiae*, Cockerell (Hemiptera: Dactylopidae)	Prickly pear, *Opuntia dillenii*	USA	1926	Goeden (1978)
Mexican beetle, *Zygogramma bicolorata*, Pallister (Coleoptera: Chrysomelidae)	Congress grass *Parthenium hysterophorus*	Mexico	1984	
Ophiomyia lantanae (Lepidoptera: Tortricidae) *Teleonemia scrupulosa* (Hemiptera: Tingidae)	Lantana weed, *Lantana camera*	Mexico	1921	Rao et al. (1971)
Pareuchates pseudoinsulata (Lepidoptera: Arctiidae)	Siam weed, *Chromolaena odorata*	West Indies	1973	Muniappan et al. (1988)
Procecidochares utilis, Stone (Diptera: Trypetidae)	Crofton weed, *Eupatorium adenophorum*	Mexico	1963	Kapoor and Malla (1978)
Cyrtobagous singularis Hustache (Coleoptera: Curculionidae)	Water fern, *Salvinia molesta*	Australia	1986	Joy et al. (1986)
Water hyacinth weevils, *Neochetina eichorniae*, Hustache *N. bruchi*, Warner (Coleoptera: Curculionidae)	Water hyacinth	USA	1983	Bennett (1984)

11.7 Future Scope of Biological Control in Pest Management

Traditional and augmentative biological controls of insect pests and weeds have a long history of achievement. An International Organization for Biological Control (IOBC) and National Bureau of Agricultural Insect Resources (NBAIR) enterprise proposed together specialist and investigators from extensively diversified fields to recognize the leading restrictions in biological control to check and to advocate method of reduction. Restrictions check to covered risk-taking strong dislike and cumbersome continuous processes, progressively administrative fence to entry of bioagents; inadequate appointment as well as conversations with peoples, colleague, farmers, and politicians of the substantial profitable satisfaction of bioagents and demoralization of biological control and its subdisciplines. In this involvement, we encapsulate a span of endorsement for incoming days that focus attention on the requirement of upgraded transmission of profitable environment and social achievement and benefits of biological control for insect pests, plant diseases, weed targeting political, administrative, farmer/land manager, and other participant interests. Governmental benefits in few countries which will be an indication of well for biocontrol in the future are discussed.

11.8 Conclusion

It is concluded that utilization of bioagents in pest management program has long as well as successful history. Now there is a different kind of obstacles, with the availability of many chances carried on with application and extensive work of beneficial insects in the pest control. Due to use of various kinds of tools in pest management provide success depend up on a climatic factor as well as give safety for human beings, development of resistance in insect pest against pesticide and rise its market price and found shortage as per people needs. Mass multiplication of bioagent and release in field for pest control are effective tools; it will overcome problem caused by insecticide in the future.

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Iron Chlorosis in Peach and Its Eco-Friendly Management: An Outlook

12

Saurabh Kumar Singh, Ramji Singh, and Ajay Tomer

Abstract

Peach [*Prunus persica* (L.) Batsch] suffers from iron chlorosis when grown in calcareous soils due to low iron availability. Traditionally, foliar and soil application of ferrous sulfate, Fe-EDTA, Fe-EDDHA chelates, etc. are adopted as corrective measures of chlorosis. Nano-fertilizer, bioremediation, and transgenic breeding approaches open innovative line of chlorosis correction. The chapter is structured to prepare a summary of the iron chlorosis: causes, detection techniques, management approaches, and future line of research. Iron fixation in calcareous soil, iron uptake by plant, advance detection techniques, and correction measures of chlorosis were explored. The significance of bioremediation and nano-fertilizers is also identified. Microbe-mediated correction measures and nano-fertilizer application are some of the eco-friendly options. Though, further research is needed in microbe-mediated correction measures and nano-fertilizer application, there is a line of research to develop Fe defficiency tolerant rootstocks through transgenic approach. These techniques will be quite useful in lowering the dependency on synthetic chemical and make better eco-friendly options for management of chlorosis in peach, as long-term solution.

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Keywords

Siderophores · Peach · *Prunus persica* · Calcareous soil · Iron chlorosis · Iron fixation

12.1 Introduction

Peach [*Prunus persica* (L.) Batsch] is one of the most common temperate region fruit crops of the world. China, Italy, the USA, Greece, Spain, Turkey, Iran, Chile, etc. are the major peach producing countries (FAOSTAT 2017). This crop belongs to the family Rosaceae. Peach (*Prunus persica* var. *vulgaris* Maxim) with round and fluffy organic product, the nectarine (*Prunus persica* var. *nectarina* (Aiton) Maxim) with round organic product however without pubescence (fluff), and the level peach (*Prunus persica* var. *platicarpa* Bailey) with level formed fruit are the three categories (Crisosto et al. 1999). Iron, the fourth most prevalent element preceded by O, Si and Al in earth crust and soils, is delegated as the fundamental micronutrient for plant development. This is multifunctional element, (Briat et al. 2007) required for different physiochemical process of plant which plays important role in chlorophyll activation, chloroplast membrane structure, photosynthesis, respiration, and synthesis of many heme proteins and in iron (Fe)–sulfur (S) clusters as helper molecules of proteins that function in fundamental life of plant (Monge et al. 1993; Balk and Pilon 2011). Higher plants utilize two general process (systems I and II) for iron securing low iron accessibility in soil (Marschner et al. 1986). Calcareous soil gives lower iron availability abreast with diminishing uptake efficiency by plant roots specially plant that depends on ferric reductase activity, because of higher soil pH and bicarbonate concentration (Jeong and Connolly 2009; Lucena et al. 2015). Out of the total 13.4 billion ha of total global land area, 1.5 billion ha of land is used for crop production which includes arable land plus land with permanent crops (Yearbook 2013). Out of total, 30% soils of the world are calcareous in nature. They are iron limiting to plant growth and development, not due to iron status in soil but because of solubility (Guerinot 2001).

Iron chlorosis in plants grown under calcareous soils is usually termed as lime-induced iron chlorosis (Pestana et al. 2003). Applications of iron containing fertilizers either in soil or as foliar spray are generally practiced to correct the iron chlorosis in peach. Kloepper et al. (1980) has given pioneer verification of iron-depriving microflora in soil, and they also reported that plant development advancing movement of plant elevating rhizobacteria pertaining to iron-chelating siderophores. These metabolites have low molecular weight and high affinity with iron (III). The involvement of siderophore and proton production resulted in improved iron bioavailability in root zone of plants (Hider and Kong 2010; Jin et al. 2013). Reports on nano-fertilizer applications are in preliminary stage. Universally accepted Fe defficiency tolerant rootstock development is in beginning, for peach. Causes, detection methods, and control measures for of Iron defficiency have been overviewed (Fig. 12.1). This chapter addresses the current trend of detection

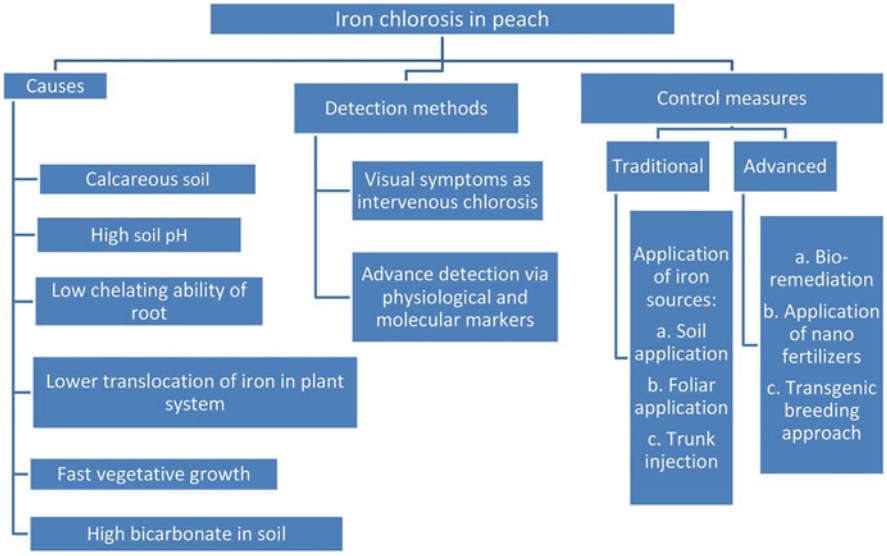

Fig. 12.1 Causes, detection methods, and control measures of iron chlorosis in peach

methods and control measures of iron cholorosis in peach and gives attention on future line of research.

12.2 Iron Fixation in Calcareous Soil

Calcareous soils have often more than 15% $CaCO_3$. Soil with high $CaCO_3$ belongs to calcisols and related calcic subgroup of other soils dominantly found in dried areas of earth (Goenaga et al. 2013). Plants show iron deficiency when developed in calcareous soil because of lower accessibility of available iron amount (Loeppert 1986). Two oxidation conditions of iron are declined structure, for example, ferrous iron (Fe^{2+}) and oxidized, for example, ferric iron (Fe^{3+}) in every single living structure. $CaCO_3$ directly takes place in the responses that diminishes iron availability to the plants. The responses of iron obsession are as per the following reactions

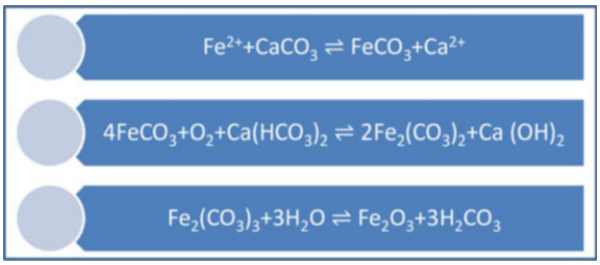

Fig. 12.2 Chemical reaction of iron fixation in calcareous soil

(Fig. 12.2). The ferrous (Fe^{2+}) is fixed as ferric oxide (Fe_2O_3) and becomes unavailable to plant roots.

12.3 Mechanism for Iron Uptake in Higher Plants

The iron uptake mechanisms of higher plant can be categorized in two groups: plant strategy and microbe mediated. In plant strategy mechanism, crops utilize both process, i.e., system I and system II of ferrous consumption (Hell and Stephan 2003), whereas microbes mediated through Fe-siderophore complexes (Kloepper et al. 1980). A brief description of uptake mechanisms is given in the following subheadings.

12.3.1 Plant Strategies for Iron Uptake

From small seasonal grain crops like rice, wheat, and so forth to the lasting tall berry crop product crops, two techniques are perceived for iron take-up. Methodology I (for non-graminaceous monocots and dicots) and system II (for graminaceous species) (Marschner et al. 1986; Jeong and Connolly 2009; Römheld 1987; Marschner and Römheld 1994).

Dicots and cereal crops utilize methodology I for ferrous isolation in the root zone of the plants. Reduction of Fe^{3+} form to Fe^{2+} form of iron at the rhizosphere increased proton (H^+) extrusion and release of reducing and/or chelating substances are the three main mechanism in the plants with strategy I (Römheld 1987). Whereas strategy II expressed except in Poaceae crop. Exudation of ferrous-binding chemicals, i.e., phytosiderophores (without amino and carboxyl peptides) from roots which aides in activating Fe-III as Fe-phytosiderophore complexes. Finally, the Fe-phytosiderophore complex is absorbed by plant root (Marschner et al. 1986). Peach suffers iron chlorosis due to lower efficiency of iron chelation at root zone in calcareous soils. Ferric chelate reductase (FC-R) ability of root can be used for Fe^{3+} tolerance screening tool (Gogorcena et al. 2005).

12.3.2 Organisms Intervened Iron Uptake

Other than systems I and II of the plants to derive iron under restricting conditions, there is likewise microbial solubilization of iron in the dirt. Proof of PGPR (plant growth promoting rhizobacteria) intervened iron bio-solubilization revealed by Kloepper et al. (1980). The number of microbes predominantly belongs to *Pseudomonas* and *Trichoderma* genera of bacterial and fungal group, respectively, has been reported for bio-solubilization of iron (Singh et al. 2019; Ravi et al. 2019). They release siderophore, like the phytosiderophores of the plants of strategy II group. Siderophore are low-molecular weight (500–1500 Da) iron-chelating compounds (Hider and Kong 2010), synthesized by microorganisms, i.e., *Pseudomonas*,

Azotobacter, *Bacillus*, *Enterobacter*, *Serratia*, *Azospirillum*, *Rhizobium*, *Trichoderma*, *Cenococcum geophilum*, and *Suillus granulatus* (Meyer and Abdallah 1978; Vinale et al. 2013). Microbial siderophores are fundamentally jumpers and low sub-atomic mass extended 200–2000 Da (Schwyn and Neilands 1987; Wang et al. 2014) compound, with particular attributes of Fe-siderophore complex development. Siderophores are typically arranged by the ligands used to chelate the ferric iron by moieties giving the oxygen ligands for Fe(III) coordination and its particular synthetic property (Das et al. 2007). The significant gatherings of siderophores incorporate the catecholates, hydroxamates, and carboxylates. The catecholates are overwhelming siderophore produce by microscopic organisms, while hydroxamate in parasites (Miethke and Marahiel 2007; Hider and Kong 2010; Schalk et al. 2011). They make stable complex with iron as Fe-siderophore solvent complex, in soil arrangement and at mineral surface, is then gets accessible for take-up by the cell layer of plant roots. Further, upon assimilation, siderophore of Fe-siderophore complexes is either recycled or destroyed (Crowley et al. 1991; Kraemer et al. 2006). Due to complex formation property of siderophores with iron as Fe-siderophore available form of soil iron, can be utilized in controlling chlorosis of peach grown in calcareous soil, has been little explored hitherto.

12.4 Markers for Advance Detection of Fe Chlorosis

Chlorophyll content (Marsh Jr et al. 1963; Smith et al. 2004) SPAD index (Smith et al. 2004; Jiménez et al. 2008), chlorophyll fluorescence (Morales et al. 1994; Lichtenthaler and Miehé 1997), thylakoid membrane lipids (Nishio et al. 1985), photosynthetic rate (Terry 1983), physiologically active iron (Oserkowsky 1933; Jacobson 1945), Fe:Mn ratio (Somers et al. 1942; Álvarez-Fernández et al. 2005), and transformed reflectance spectra (Li et al. 2006) are important physiological parameters used for the detection of iron chlorosis in different crops. Literature supports the prospects of using these markers, viz., physiological and molecular as advance detection technique of iron chlorosis.

12.5 Physiological Markers

Brown (1956) emphasized to study the biochemical basis of iron chlorosis and its contributing factors. The efficiency of iron uptake depends on plant species (Christ 1974). Iron status of different plant parts like leaves, bark, flowers, vegetative buds, and floral buds has been reported to use as index tissue in different crops for predicting the iron chlorosis. Floral analysis is reported as a tool for prediction of iron deficiency in peach (Sanz et al. 1996, 1997).

Iron plays important role in chlorophyll formation (Marschner 2011; Briat et al. 2015; Kaya and Ashraf 2019). The lessening in the number of granal and stromal lamellae and in the number of thylakoids under iron stress condition was reported by Terry (1980). In parallel, Terry (1980) also reported diminish in chlorophyll (Chl) a

and Chl. b contents of sugarbeat (*Beta vulgaris* L.) leaves under Fe stress condition but had no effect on the number of chloroplast per unit area. Quantitative reduction (75%) in chlorophyll content per unit area and the role of iron in chloroplast development were also noted in sugarbeat (Taylor et al. 1982; Nishio and Terry 1983). Findings showed that there is a quantitative decrease in chlorophyll content of leaves under iron stress condition. Chlorophyll fluorescence and iron concentration in flowers of the peach, root apoplastic iron in soybean, and morphological changes of plant root coupled with alteration in citrate concentration in phloem in castor bean are found directly correlated with chlorosis under iron stress condition (Longnecker and Welch 1990; Schmidke et al. 1999). So, chlorophyll content of leaves, SPAD index reading, chlorophyll fluorescence, concentration of iron in plant parts, and change in root morphology can be used as marker for advance detection of iron stress. These predictions will be helpful to manage the iron chlorosis in peach. Foliar iron application could be used for remediation of chlorosis problem (Abadía et al. 2001). Nicotianamine (A non-proteinogenic amino acid), nitric oxide levels, and concentration of nutrients in reproductive buds need extensive research to be used as marker for the selection of Fe-efficient genotypes in *Prunus* sp. (Pich and Scholz 1996; El-Jendoubi et al. 2012).

12.6 Molecular Markers

The need for search blueprint of iron transport, molecular mechanism of genes controlling iron uptake, and intracellular storage was emphasized by Briat and Lobreaux (Briat and Lobréaux 1997). Current researches clarified that in different microorganism, a small regulatory RNA, RyhB, plays essential role in metabolism of iron. Numerous data published at molecular level of iron transport in plant need comprehensive research on iron homeostasis (Masse et al. 2007; Curie and Briat 2003). *Arabidopsis thaliana* (arabidopsis), *Lycopersicon esculentum* (tomato), and *Pisum sativum* (pea) are used as model plants to study strategy I of iron absorption. Iron is translocated as ferric citrate complex in xylem with the help of FRD3 effluxes citrate, from the root to shoot portion of plants (Durrett et al. 2007). A lot of information for molecular basis of iron transport and compartment has been decoded. There is a need to spell out each Fe translocation step, iron chelator complexes, Fe flux, signals, and receptors regulating Fe nutritional status (Kobayashi and Nishizawa 2012; Balk and Schaedler 2014). Fe is concentrated in the vacuoles of cells. A group of co-expression genes is involved in iron deficiency regulation (Mary et al. 2015; Li et al. 2015). In iron translocation, there are functional links between loading in vacuoles (AtVIT1 gene) and remobilization (AtVIT1 and AtNRAMP3 genes) in *Arabidopsis*, and iron accumulation in vacuolar globoids is obstructed with mystifying genes (Mary et al. 2015). Gonzalo et al. (2011) studied P 2175 (Myrobalan plum) and Felinem (peach-almond hybrid) for differential expression of genes involved in homeostasis. Genes, PFRO2 (for reductase activity), PIRT1 (for transport in roots), and PAHA2 (for proton release), were expressed, which can be used as molecular marker in screening and developing

cultivar as well as rootstock for iron tolerance in fruit crops. Molecular advancement of iron regulation and decoding of iron regulatory gene will be helpful in peach for managing iron chlorosis.

12.7 Chlorosis Control Measures in Peach

12.7.1 Index Tissue

Based on nutrient status of specific plant part, the fertilizer rate may be recommended to correct nutritional deficiency. In sampling, the age of selected plant part and time of sampling should be considered. Concurrently, avoid the sampling from plants damaged due to insect pest infestation, pathogen attacks, mechanical injuries must be avoided. Details of plant analysis principles, sampling procedure, and laboratory analysis are given by Jones (1983). In peach, leaves near current year growth should be sampled during mid-season of growth, with a sample size of 50–100 selected plants. Best sampling time in peach with correlation to yield was found at 60 days after full bloom (Sauz et al. 1992).

12.7.2 Exogenous Application of Iron Sources

Soil and foliar application of synthetic iron sources are used for controlling iron chlorosis in peach. The later practices are quite effective (Hergert et al. 2019; Ma et al. 2019; Sharma et al. 2019; Tan et al. 2019). Though, the variable responses of exogenously applied iron are recorded. So, the foliar application of iron cannot yet be deem an effective strategy to control the problem.

12.8 Future Lines of Research

12.8.1 Bioremediation

Foliar application of Fe is a widespread strategy to manage lime-induced iron chlorosis (Abadía et al. 2001; Mortvedt 1991; Pal et al. 2019; Niyigaba et al. 2019). However, reports indicated variable responses to Fe sprays and foliar application of iron to manage chlorosis cannot yet be considered a reliable strategy to mitigate plant Fe deficiency (Tagliavini et al. 2000; García-Laviña et al. 2001). Soil applications of iron sources have its own limitation. Due to its oxidized form as ferric state in soil, it forms very insoluble minerals. In addition to their practical applicability intricacy, these chelated chemicals are too expensive. Due to limitation of application of iron source, microorganism-mediated bioavailability of iron can be an effective way to control iron chlorosis.

Crowley et al. (1988) confirmed the presence of a microbial siderophore iron vehicle framework in oat (*Avena sativa* cv. Victory). Application of bacterial

siderophore of the two siderophore-producing bacterial strains, namely, *Chryseobacterium* spp. C138 from the root zone of *Oryza sativa* and *Pseudomonas fluorescens* N21.4 from the root zone of *Nicotiana glauca*, in iron-starving tomato plants grown in hydroponic system resulted significantly higher plant yield, chlorophyll, and iron content (Radzki et al. 2013). The finding indicated that bacterial siderophores are helpful in supplementing iron to plant (Naafi and Rahayu 2019, Nogiya et al. 2019). Another experiment on red bean under greenhouse condition showed increase in bean plant growth factors significantly, inoculated with 7NSK2, UTPF5, and UTPF 76 strains of fluorescent *Pseudomonas* (Omidvari et al. 2010). The beneficial effect of microbial siderophores has a potential to correct lime-induced chlorosis in peach.

12.8.2 Application of Nano-Fertilizers

Experiments have been planned and executed to analyze the penetration, translocation, and effect of nanoparticles on crop plants (Corredor et al. 2009; Kukde et al. 2019; Tong et al. 2018). Results were in support of practical utility of nanoparticle due to the ability of penetration and translocation in plant tissues. Carbon-iron magnetic nanoparticles have shown potential as a matter of fact but need more research and refinement (Corredor et al. 2010). The importance of nanomaterial in context to eco-friendly approach is also reviewed (Ghormade et al. 2011; Chhipa 2019). Foliar application with *Ascophyllum nodosum* extricate and nano-iron chelate manure on a number of branches per plant, number of natural product per plant, organic product size, and organic product yield were estimated, at the hour of development. Improvement was noted with the use of *A. nodosum* remove and nano- iron chelate manure (Bozorgi 2012). The impact of foliar and soil application of iron oxide nanoparticles on root growth and photosynthetic parameters was deliberated. There were higher root elongations compared to bulk counterpart. The photosynthetic rate was also increased significantly (Alidoust and Isoda 2013). Different types of nanoparticles, Viz., nano ZnO, nano FeO, and nano Zn, CuFe-oxide were used on hydroponically grown mung (*Vigna radiata*) seedling. All treated plant showed increased growth over control, although nano-Zn, CuFe-oxide was best. The absorbance of nanoparticles was also detected (Dhoke et al. 2013). Potential application of nanoparticles in sustainable agricultural practices to reduce environmental pollution has been outlined by several authors (Prasad et al. 2014, 2017a, b; Sekhon 2014). Applications of nanoparticle -based fertilizers in agriculture enhance the availability of nutrients as nano-fertilizers (Servin et al. 2015) and strengthen the possibility that nano-fertilizers have supremacy over traditional control measures of chlorosis in peach.

12.8.3 Rootstock Breeding and Transgenic Technology

There is not universally accepted iron-tolerant rootstock in peach. The efforts made with traditional breeding has been unable to escalate progress. Introducing novel genomic regions directly to recipients can help in achieving success in improvement programs triggered new interest. There are reports on successfully regenerating peach transgenic plants by using immature embryo as explants (Smigocki and Hammerschlag 1991), genetic transformation through biolistic method (Ye et al. 1994), and compact growth habit possibility by altering *ipt* gene (Hammerschlag et al. 1996). Methodology to develop transgenic peach plants round the year by using embryonic axis sections as explants from stored mature seeds (Pérez-Clemente et al. 2005), and optimum conditions for novel gene introduction to explants via *Agrobacterium*-mediated gene transfer (Padilla et al. 2006) witnessed success in transgenic development in peach. These findings directed toward the possibility to develop Fe deficiency-tolerant rootstocks for peach grown in calcareous soils.

12.9 Conclusion

Peach is unexplored in terms of application of nano-fertilizer and bioremediation. It is in this manner that it is important to assess the reaction of nano-fertilizers and microorganism for controlling iron chlorosis in peach, grown in calcareous soils. Microbial iron mobilization needs vast research for identifying efficient strains regarding iron mobilization and their effect on plant growth, nutritional status, and yield. There is immense scope of research to develop Fe-tolerant rootstocks that have universal acceptability. In the future, alternative measures viz., bioremediation, nano-fertilizer application, and use of iron-tolerant rootstocks will help in reducing dependency on chemical measure of controlling chlorosis in addition to eco-friendly remediation as long-term solution.

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Role of Microbes in Plastic Degradation

13

Garima Singh, Sonal Yadav, Kanika Chowdhary, and Satyawati Sharma

Abstract

Conspicuous usage of plastics at enormous scale in packaging industry in the last three decades has resulted into a colossal problem that is yet to be resolved by researchers and related stakeholders. The egregious level of environmental persistence of plastic polymers and their byproducts has deleterious effects on living beings (plants and animals). Anthropologists have projected that discarded plastics will outnumber fishes in ocean, if the menace remains unmanageable. Microbes are the backbone of all bioconversion processes taking place in ecosystems. They have the ability to thrive in extreme conditions due to their impressive enzymatic machinery. Biodegradation by microorganisms can be carried out either aerobically or anaerobically. Different bacterial and fungal species (including fungal endophytes) are perceived as prospective solution for plastic biodegradation. The present chapter highlights the various investigations describing efficient usage of plastic degrading microbes.

Keywords

Microbial decomposers · Fungal endophytes · Biodegradation · Serine hydrolases · Biofragmentation

13.1 Introduction

India consumes approximately 4% of plastics produced globally and is estimated to be a leading consumer in Asia-Pacific region with an escalating demand of nearly eight million tons by 2023 (Grover et al. 2015). Plastic wastes are remarkably

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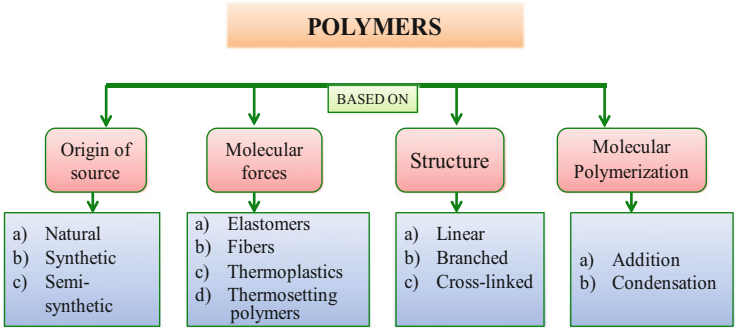

Fig. 13.1 Classification of polymers (Adapted from: https://nptel.ac.in/courses/104103071/39)

high-persistent materials, which can cause deleterious effects on ecosystem (i.e., soil infertility, depletion of underwater source). Polyethylene was an immediate hit commodity among masses soon after its launch due to its durability, high resistant toward multiple chemicals, and cheap production. LDPL (low-density polyethylene) used as multi-utility use and throw carry bags makes for the larger chunk of plastic discard (Skariyachan et al. 2015). Following polyethylene, next most used polymer is polystyrene. German-based chemist named Eduard Simon discovered it in 1839 as a monomer from a tree resin, while Hermann Staudinger described its polymeric property for the first time (Ogunsona et al. 2011).

Partially decomposed plastics produce highly toxic halogenated compounds known as persistent organic pollutants (Jayasekara et al. 2005), which bioaccumulates in animal tissues due to high lipid solubility. Plastic is disposed through different physical and chemical processes, i.e., incineration, landfilling, and recycling. Persistent organic pollutants (POPs) are globally distributed after being released into air, water bodies, and soil. They enter into animal food chain via ingestion of contaminated sea foods and due to lipophilicity get deposited into adipose tissues. They travel extreme distances throughout earth via atmospheric processes including industrial sites and agricultural areas.

Polymers are the substances formed through joining together of smaller recurring structural unit's molecules called monomers. The subsequent molecule holds larger molecular mass in comparison to precursor molecules, having distinctive physical properties such as durability, viscosity, elasticity, and crystallinity. Classification of polymers is done based on their different properties (i.e., chemical, mechanical, and physical properties) and technological application for which they are aimed at (Cowie 1991) (Fig. 13.1). Different categories of synthetic polymers in use by the society at large are enlisted (Table 13.1). Because of their wide-ranging applicability, both synthetic and natural polymers are vital part of our everyday life activities. According to an estimate, almost 140 MT of synthetic polymers are created worldwide annually (Leja and Lewandowicz 2010).

The highest utility of plastic is in packaging industries which is increasing enormously. It has been a handy substitution for paper-based packages due to enhanced stability and durability. Inappropriately discarded plastic polymers are

Table 13.1 Various types of synthetic polymers

Polyethylene	Plastic bags, packaging, and toys
Polystyrene	Disposable cutlery, laboratory, and packaging of food items
Polyurethane	Footwear, cushion materials, furniture bedding, and insulators
Polyvinyl chloride	Automobile seat covers, housing and constructions, pipe fittings
Polypropylene	Sportswear, laboratory and industrial equipment, medical devices
Polytetrafluoroethylene	Electronics, nonstick kitchenware, moisture-resistant furniture and wearables
Polycarbonate	Optical lenses and reflectors, street lighting, rear lights in vehicles, bullet proof shields, swimming goggles

found as gigantic dumps in municipal solid waste, landfills, and marine waters highlighting the challenge of dealing with their non-biodegradability which might persist for centuries to come. Even in most advanced cities of India, solid waste management is an undervalued task, and nearly 90% of it is dumped openly. And this waste is further disposed through unregulated methods (i.e., landfilling, composting, and incineration). The proponents of circular economy advise on resourcefully recovering the disposed plastic and recycle it extracting its value again. Many degradation techniques have been applied, but biological degradation technique is ecofriendly and cheaper than chemical and thermal degradation. Biodegradation of plastic is carried out by microorganism or microbial consortium which is able to utilize plastic polymers as a sole carbon source and convert it nontoxic products and energy.

13.2 Biodegradation of Polymers

The polymer degradation generally occurs by two methods, i.e., abiotic and biotic (Fig. 13.2). Photooxidation is basically deterioration of polymeric organic matter via UV region of solar radiation and results into low molecular weight fragments and

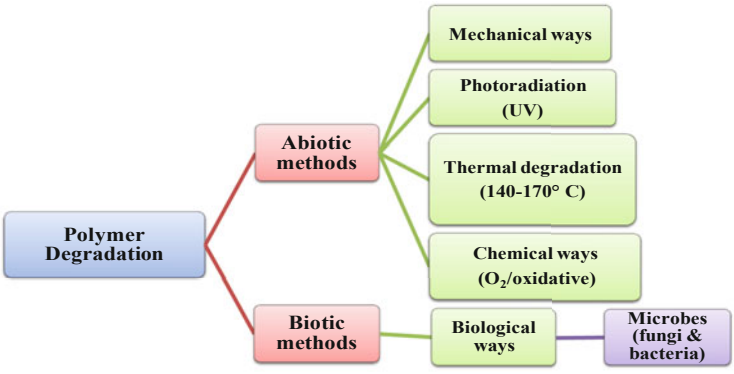

Fig. 13.2 Degradation of polymers in aerobic and anaerobic conditions (Adapted from: Gu 2003)

radical production (Arkatkar et al. 2009). The biodegradation always follows photodegradation and chemical degeneration (Shah et al. 2008). Microorganisms are the backbone of all bioconversion processes taking place in ecosystems. They are responsible for the biodegradation of organic matter both directly and indirectly. Biodegradation process by microorganisms can be broadly divided into two types, namely, aerobic biodegradation and anaerobic biodegradation (Gu 2003). Enzymatic machinery of the potent plastic degenerating microbes has the instrumental role in the entire process. Various fungal and bacterial species have been found to function as biological means occur naturally and have dissimilar degradation abilities for polymers.

Filamentous fungi of basidiomycetes, ascomycetes, and zygomycetes classes have been widely mentioned as biocatalysts in literature. Some of the noted genus of fungi, which are found effective in the biodegradation process, are *Talaromyces* sp., *Sporotrichum* sp., *Trichoderma* sp., *Fusarium* sp., *Aspergillus* sp., *Gliocladium* sp., *Penicillium* sp., *Phanerochaete* sp., *Penicillium* sp., *Paecilomyces* sp., *Ganoderma* sp., *Geotrichum* sp., *Candida* sp., *Thielavia* sp., *Thermoascus* sp., *Geotrichum* sp., *Phlebia* sp., *Thermomyces* sp., *Geotrichum* sp., *Cladosporium* sp., and *Trametes* sp. (Tuomela et al. 2000; Delort and Combourieu 2001). Barratt et al. (2003) reported *Geomyces pannorum* as most efficient polyurethane degrading fungus isolated from soil samples buried polyester for over a month (Barratt et al. 2003). The PU degradation depicted by the fungus ranged between 22% and 100%. This study for the first time described correlation between water holding capacity of soil and PU fungal strains.

Microbial decomposers disintegrate biodegradable material into tiny subfractions through biodeterioration by causing alteration in physical and chemical properties in the polymer (Walsh 2001; Eggins and Oxley 2001). These catalytic microbes secrete hydrolyzing enzymes, which are able to cleave larger polymeric molecules into smaller molecular units. The intracellular molecules integrate and assimilate in microbial metabolism to produce energy and several primary and secondary metabolites. Concurrently, extracellular oligomers are further oxidized into other smaller organic compounds and produce carbon dioxide, nitrogen, methane, and water as byproducts. This extracellular process is called mineralization (Fig. 13.3). Polymer mineralization could occur both aerobically and anaerobically. In aerobic condition carbon dioxide and water are formed, while under anaerobic conditions, methane is also released. Well-known bacteria species capable of polymeric degradation are *Pseudomonas aeruginosa*, *Comamonas acidovorans*, *Streptomyces badius*, *Rhodococcus ruber*, and *Clostridium thermocellum* (Singh and Sharma 2008).

13.2.1 Mechanism and Pathways Involved in Polymer Degradation by Fungus

Polymer degradation by fungus depends largely on the hydrolyzing enzymes secreted by it. In addition to this, it is ruled by several factors such as the

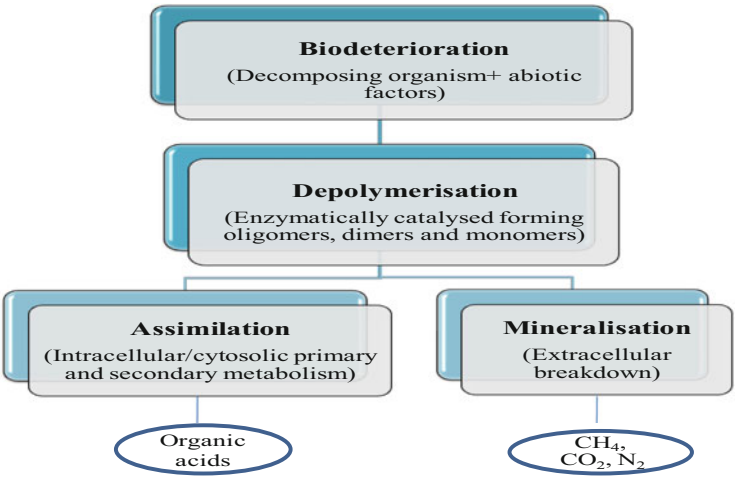

Fig. 13.3 Mechanism of biological degradation of polymers (Adapted from: Lucas et al. 2008)

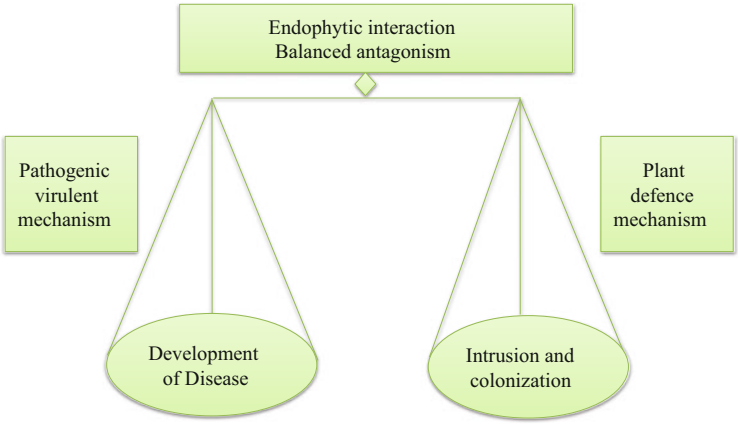

Fig. 13.4 Balanced antagonism hypothesis of endophytes (Adapted from: Schulz et al. 1999)

environment (in situ or ex situ), structure of the pollutant, and ionization potential (Kadri et al. 2017).

Fungal endophytes have been recognized as intriguing group of organisms which have the ability to colonize plant internal tissues asymptomatically. They synthesize numerous bioactive compounds including phytohormones. The hypothesis explaining asymptomatic presence of endophytes as balanced antagonism is depicted in Fig. 13.4 (Schulz and Boyle 2005). Endophytic fungi have been recognized as a prospective reservoir of highly useful and functional enzyme producers that can be utilized in induction of polymer degradation. Endophytic mycopopulation of two Indian indigenous plants, namely, *Humboldtia brunonis*

and *Psychotria flavida*, were cultivated on heap plastic films and investigated for their plastic biodegrading ability. The isolated fungi were grown on irradiated polypropylene (PP) and polythene (PE) films as sole carbon source for 3 months. The pattern of biodegradation by different fungi was examined by studying empirical modifications in average molecular weight and viscosity through SEM, FTIR spectroscopy, and differential scanning calorimetry. It was found that *Lasiodiplodia theobromae* and *Aspergillus* sp. recovered from *P. flavida* and *Paecilomyces lilacinus* of *H. brunonis* could biodegrade irradiated polypropylene film effectively (Sheik et al. 2015). Likewise, Raghavendra et al. (2015) characterized famous medicinal plant *Azadirachta indica* for presence of polyurethane (PUR) degrading endophytes in vitro in both solid and submerged media. *Fusarium* sp. was found as best candidate in both aerobic and anaerobic conditions. Similarly, stem tissues of woody plants of *Ecuadorian Amazonian* rainforest were randomly explored for PUR-degrading fungi. *Pestalotiopsis* sp. isolates exhibited strong activity. Enzymatic protein having molecular mass of 21 kDa belonging to serine hydrolase family caused considerable PUR degradation (Russell et al. 2011).

13.2.2 Degradation of Polymeric Wastes by Bacteria

Several bacterial species (listed in Tables 13.2 and 13.3) have the ability to degrade synthetic plastics along with the natural plastics (Gu 2003). The advantage seen with case of bacteria is that they can degrade plastic in both anaerobic and aerobic conditions (Chandra & Rustgi 1998; Kumar et al. 2011). According to some researchers, 17 genera of bacteria have been reported till now that have the ability to degrade plastics (Hugenholtz et al. 1998). Plastic degradation is initiated by the hydrolysis or oxidation through bacterial enzymes that breakdown the large compound chain into smaller (monomer) via metabolic method. With the aid of proper availability of water, soil type, redox potential, temperature, carbon, and energy source, bacterial growth can be maintained leading to the reduction in plastic in an eco-friendly manner (Sand 2003). It has been seen that microorganisms identify polymers as a source of the organic compound (Premraj and Doble 2005).

Pseudomonas sp., *Bacillus megaterium*, *Halomonas* sp., *Ralstonia eutropha*, *Azotobacter* sp., etc. have been used to degrade plastics (Chee et al. 2010), and *Bacillus brevis*, *Acidovorax delafieldii*, *Paenibacillus amyloticus*, *Bacillus pumilus*, *Bordetella petrii*, *Pseudomonas aeruginosa*, and *Shewanella* sp. (Uchida et al. 2000; Teeraphatpornchai et al. 2003; Hayase et al. 2004; Kim and Park 2010; Lee and Kim 2010; Sekiguchi et al. 2011) have been found to degrade bioplastics. A large number of bacteria have been testified to degrade polyethylene, namely, *Acinetobacter baumannii*, *Viscosus* spp., *Thuringiensis*, *Bacillus amyloliquefaciens*, *Arthrobacter viscosus*, *B. mycoides, B. cereus, B. pumilus*, *Staphylococcus cohnii*, *Xylosus* spp., *Rahnella aquatills*, *Pseudomonas* spp., *M. lylae*, *Paenibacillus macerans*, *Pseudomonas fluorescens*, *Flavobacterium* spp., *Delftia acidovorans*, *Ralstonia* spp., *Arthrobacter* sp., *Rhodococcus erythropolis*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, and *Bacillus brevis* (Watanabe et al. 2009; Koutny et al. 2009).

Table 13.2 List of different bacteria associated with polymer degradation

S. no.	Microorganisms	Types of plastic	Microbial source	Biodegradation efficiency (%)	References
1.	*Bacillus cereus*	Polyethylene/polythene (PE)	Soil (dumpsite)	7.2–2.4	Sowmya et al. (2014)
2	*Pseudomonas putida*	Milk cover	Soil (garden)	75.3	Saminathan et al. (2014)
3	*Pseudomonas* sp.	Natural and synthetic polyethylene	Sewage sludge dumpsite	31.3 and 16.3	Nanda et al. (2010)
		Polyethylene and plastic	Soil (mangrove)	20.54 and 8.16	Kathiresan (2003)
5	*Micrococcus luteus*	Plastic cups	Soil (forest)	38	Sivasankari and Vinotha (2014)
6	*Masoniella* sp.			27.4	
7	*Rhodococcus ruber*	Branched low density (0.92 g/cm^3) polyethylene carry bags	Not specified	7.5	Sivan et al. (2006)
8	*Pseudomonas stutzeri*	LDPE and polyethylene	Soil from plastic dumping sites	73.38	Sharma and Sharma (2004)
9.	*Pseudomonas* sp. and *Arthrobacter* sp.	HDPE	Dumped sites	15 and 12, respectively	Balasubramanian et al. (2010)
10.	*Bacillus cereus*	LDPE	Municipal compost yard	17.036	Pramila and Ramesh (2011)

Table 13.3 Microorganisms associated in degradation of different types of polymers

Types of polymers	Microorganisms	Applications	References
Polyethylene	*C. cladosporioides*, *F. redolens*, *Fusarium* spp., *P. simplicissimum* YK, *P. simplicissimum*, *P. pinophilum*, *P. frequentans*, *P. chrysosporium*, *V. lecanii*, *G. virens*, *M. circinelloides*, *A. kiliense*, *B. borstelensis*, *Comamonas acidovorans*, *Pseudomonas chlororaphis*, *Phanerochaete chrysosporium*, *P. aeruginosa*, *Rhodococcus erythropolis*, *R. rubber*, *R. rhodochrous*, *S. cohnii*, *S. epidermidis*, *S. xylosus*, *Streptomyces badius*, *S. setonii*, *B. amyloliquefaciens*, *B. brevis*, *B. cereus*, *B. circulans*, *B. halodenitri cans*, *B. mycoides*, *B. pumilus*, *B. sphaericus*, *B. thuringiensis*, *Arthrobacter para neus*, *A. viscosus*, *Acinetobacter baumannii*, *M. paraoxydans*, *Nocardia asteroides*, *M. luteus*, *M. lylae*	Drink bottles, peanut butter jars, plastic films	Kale et al. (2015); Restrepo Flórez et al. (2014); Bhardwaj et al. (2012)
Polyvinyl chloride (PVC)	*Pseudomonas fluorescens* sp., *P. putida*, *P. chlororaphis*, *A. niger*	Plumbing pipes and guttering, shower curtains, window frames, flooring	Bhardwaj et al. (2012), Kale et al. (2015)
Polyethylene	*Cladosporium cladosporioides*, *Fusarium redolens*, *Fusarium* spp. AF4, *Penicillium*	Drink bottles, peanut butter jars, plastic films	Kale et al. (2015); Restrepo Flórez et al. (2014);

(continued)

Table 13.3 (continued)

Types of polymers	Microorganisms	Applications	References
	simplicissimum YK, *P. simplicissimum*, *P. pinophilum*, *P. frequentans*, *Phanerochaete chrysosporium*, *Verticillium lecanii*, *Gliocladium virens*, *Mucor circinelloides*, *Acremonium kiliense*, *Phanerochaete chrysosporium* *Brevibacillus borstelensis*, *Comamonas acidovorans*, *Pseudomonas chlororaphis*, *P. aeruginosa*, *P. fluorescens*, *Rhodococcus erythropolis*, *R. rubber*, *R. rhodochrous*, *Staphylococcus cohnii*, *S. epidermidis*, *S. xylosus*, *Streptomyces badius*, *S. setonii*, *S. viridosporus*, *Bacillus amyloliquefaciens*, *B. brevis*, *B. cereus*, *B. circulans*, *B. circulans*, *B. halodenitri cans*, *B. mycoides*, *B. pumilus*, *B. sphaericus*, *B. thuringiensis*, *Arthrobacter para neus*, *A. viscosus*, *Acinetobacter baumannii*, *Microbacterium paraoxydans*, *Nocardia asteroides*, *Micrococcus luteus*, *M. lylae*		Bhardwaj et al. (2012)
Polyvinyl chloride	*Pseudomonas fluorescens* B-22, *P. putida* AJ, *P. chlororaphis*, *Ochrobactrum* TD, *Aspergillus niger*	Plumbing pipes and guttering, shower curtains, window frames, flooring	Kale et al. (2015); Bhardwaj et al. (2012)

(continued)

Table 13.3 (continued)

Types of polymers	Microorganisms	Applications	References
Polyurethane	*Exophiala jeanselmei*, *Trichoderma* spp., *Pestalotiopsis microspore*, *Curvularia senegalensis*, *Fusarium solani*, *Aureobasidium pullulans*, *Cladosporium* spp., *Trichoderma* DIA-T spp.	Fibers, foams, paints, coating, packaging	Kale et al. (2015); Bhardwaj et al. (2012); Owen et al. (1996)
Poly (3-hydroxybutyrate) PHB	*Alcaligenes faecalis*, *Aspergillus fumigatus*, *Pseudomonas lemoignei*, *Penicillium* spp., *Schlegelella thermodepolymerans*, *Penicillium funiculosum*	Pharmaceutical and drug delivery	Kale et al. (2015); Bhardwaj et al. (2012)
Polycaprolactone	*Amycolatopsis* spp., *Aspergillus flavus*, *Bacillus brevis*, *Clostridium acetobutylicum*, *Clostridium botulinum*, *Fusarium solani*	Implantation and controlled drug release	Shah et al. (2008); Bhardwaj et al. (2012)
Polylactic acid	*Bacillus brevis*, *Penicillium roqueforti*, *Amycolatopsis* spp.	Food handling and medical implants	Shah et al. (2008)
Polyesters	*Aspergillus terreus*, *Bionectria* sp. E2910B, *Chaetomium globosum* *Cladosporium* sp., *Curvularia senegalensis* *Fusarium solani*, *Lasiodiplodia* sp. E2611A, *Pestalotiopsis microspore* E3317B, *P. microspora* E2712A, *Pleosporales* sp. E2812A, *Pseudomonas* sp., *Tenacibaculum*, *Alcanivorax*, *Mesorhizobium* sp., *Xanthomonadaceae* sp., *Pseudomonas chlororaphis*	Pillow material for insulation and cushioning; coating of fabrics, comforter upholstery padding, etc. Car tire reinforcements; fabrics for safety and conveyor belts; cushioning applications for furniture, car bedding seats, carpet, etc.	Russell et al. (2011); Crabbe et al. (1994)

(continued)

Table 13.3 (continued)

Types of polymers	Microorganisms	Applications	References
Polyesters and polyethers	*Penicillium funiculosum*, *C. globosum*, *Aspergillus flavus* *Aspergillus niger*, *Aspergillus versicolor*, *A. pullulans*, *Trichoderma* spp.		Darby and Kaplan (1968)
Polyethers	*E. jeanselmei* REN-11A		Owen et al. (1996)

The advantage of bacteria that produce biofilms is more efficient than planktonic strains in degrading plastic. Bacterial biofilms comprise extracellular polymeric substances (EPS) that play a major role in biofilm formation and stability (Gilan and Sivan 2013). Some species of Gram-negative (*Pseudomonas fluorescens*, *E. coli*, *Vibrio cholera*, and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus epidermidis*, *Staphylococcus enterococci*, and *Staphylococcus aureus*) were broadly described for biofilm formation. However, several microorganisms have also been reported generally in biofilms formation (O'Toole et al. 2000; Prakash et al. 2003). The *Pseudomonas* sp. degraded low-density polyethylene (LDPE) by 5 ± 1% by the formation of biofilm on its surface. The formation of biofilm depends upon the adaptable nature of bacteria. The *Pseudomonas* sp. shows degradation of LDPE by means of enhanced hydrolytic activity (31%) and cellular surface hydrophobicity (~26%). It has been observed that tween 80 had negative effects on hydrophobicity (Tribedi and Sil 2013; Tribedi et al. 2015). Sivan et al. (2006) isolated the strain *Rhodococcus ruber* C208, a biofilm-producing bacterium that has 0.86% biodegradation rate per week. It has also been reported that degradation rate with *R. ruber* C208 can be enhanced by 50% using mineral oil and augment the biofilm prospective by growing microbial colonization on polyethylene surface (Orr et al. 2004).

A natural polymer, i.e., poly (cis-1,4-isoprene), is produced by nurturing and tapping the *Hevea brasiliensis* (rubber tree). According to earlier reports, *Xanthomonas* sp. was the only identified Gram-negative bacterium that has the capability to consume this natural rubber (Bode et al. 2000) and utilize it as a sole carbon and energy source (Tsuchii and Takeda 1990). However, the recent study explored that the species like *Pseudomonas citronellolis and Streptomyces coelicolor* the have the ability to degrade synthetic poly(cis-1,4-isoprene) along with the vulcanized rubber.

13.2.3 Factors Affecting Degradation of Polymers

Decomposition of the polymer depends on chemical configuration which upkeeps the development of microorganisms in the form of nutrient sources. The chemical structure (responsible for functional group stability, hydrophilicity, and reactivity) is the utmost significant factor affecting the biodegradability of polymeric materials. Other significant aspects are properties, e.g., molecular weight and morphology (crystalline, amorphous), porosity, and elasticity (Acemoglum 2004; Anderson and Shive 1999). Discoloration, phase separation, cracking, and erosion are some of the characteristics which indicate the degradation of polymers. Breakage of bonds, transformation due to chemicals, and synthesis of new functional groups are responsible for the variations (Bhardwaj et al. 2013). Polymer made from starch or flax fiber shows greater biodegradability as compared to other synthetic polymers. Starch-based polymer is favorable for microbial attack, and hydrolytic enzymes act on the polymer matrix to reduce their weight (Kumar et al. 2011; Sen and Raut 2015). Apart from that, several external factors such as temperature and chemicals also enhance the rate of degradation of polymers by microbes (Bhardwaj et al. 2012).

13.3 Involvement of Enzymes Secreted by Microorganisms in Biodegradation Process of Polymer Wastes

Biochemical transformation by microorganisms helps in reduction of structural complexity of different polymers. Breakdown of molecules into simpler forms plays an essential role in reduction of toxicity. Microorganisms augment the biodegradation route by secreting certain catalytic enzymes (Hadad et al. 2005). This microbial route serves an integral role for management of plastic wastes generated, thereby providing a sustainable solution to increasing environmental problems owing to presence of plastics (Singh and Sharma 2008).

Enzymes secreted by microorganisms are substrate specific. Hence, biodegradation pathway undertaken varies according to the substrate and enzymatic reactions (Underkofler et al. 1958). Microbial enzymes serve as an essential source to hydrolyze low molecular weight components as well as soluble macromolecules (Gallert and Winter 2005). The rate of biodegradation of polymers by microbial route is governed by factors such as oxygen supply, nutrient access to microbes, optimal states for enzymatic reactions such as temperature, compound's structure, pH, and cellular transport process (Singh and Ward 2004). The biodegradation process of fungi is greatly enhanced under aerobic conditions (Kumar et al. 2011).

Fungi own an intrinsic ability to degrade polymers (Li et al. 2014). Ligninolytic enzymes secreted by fungus are best reported for production of extracellular enzymes accountable for degrading polymers (Zhang et al. 2015). Ligninolytic system comprises three principal groups of enzymes, namely, peroxidases such as lignin and manganese and phenol oxidase such as tyrosinase and laccase (Novotný et al. 2004). Dioxygenases, proteases, cytochrome P_{450} monooxygenase, lipases,

Table 13.4 Enzymes secreted by Fungus responsible for poly-aromatic hydrocarbons degradation

Fungus	PAHs	Enzymes involved	Metabolites	References
Phanerochaete chrysosporium	Phenanthrene	Manganese peroxidase and lignin peroxidase	2,2′-diphenic acid	Cerniglia and Yang (1984)
Phanerochaete chrysosporium	2-methylnaphthalene, [^{14}C]benzopyrene and [^{14}C] biphenyl	Manganese peroxidase and lignin peroxidase	^{14}CO2	Haemmerli et al. (1986) Lee et al. (2010)
Pleurotus ostreatus	Phenanthrene	Hydrolase, cytochrome P_{450}, and monooxygenase epoxide	2,2′-diphenic acid and phenanthrene trans-9,10-dihydrodiols	Bezalel et al. (1997)
Phanerochaete chrysosporium	Phenanthrene	Non-lignolytic enzymes	Phenols and trans-dihydrodiols	Cerniglia and Yang (1984)
Polyporus sp. *S133*	Fluoranthene, phenanthrene, fluorene, anthracene, and pyrene	Peroxidase and de-oxygenase	Benzoic acid, catechol, and phthalic acid	Hadibarata et al. (2012)

and epoxide hydrolases have also been significantly reported for their biodegradation capability for polyaromatic hydrocarbons (Balaji et al. 2014).

Lipase enzyme secreted by *Rhizopus delemar* is reported in literature for the biodegradation process of polylactic acids with low molecular weight (Masaki et al. 2005). *P. chrysosporium*, *Polyporus* sp. S133, *B. adusta*, and *Pleurotus ostreatus* have been reported for metabolism process of PAHs (Haritash and Kaushik 2009). *P. chrysosporium* is reported for efficient biodegradation of polyethylene (Shimao 2001). Different studies document that higher oxidative potential increases PAHs degradation in latter phase of enzymatic reactions by microbes (Bezalel et al. 1997). Degradation of polyaromatic hydrocarbon phenanthrene with different metabolites secreted in the process is summarized in Table 13.4.

Plastics obtained from renewable resources such as polylactic acid (PLA) can be used further by employing the recycling process. Polyurethane esterase secreted by *Comamonas acidovorans* and lipase enzyme secreted by *R. delemar* are reported for the biodegradation of PLAs having low molecular weight, whereas high molecular weight PLAs have been reported to be biodegraded with bacterial strain *Amycalotopsis* sp. (Dashtban et al. 2010).

Different enzymes (esterases, lipases, proteases, and ureases) have been documented for cleavage of ester bonds, thus playing an essential role in biodegradation of polyurethane (Russell et al. 2011). Lipase, esterase, and serine hydrolase

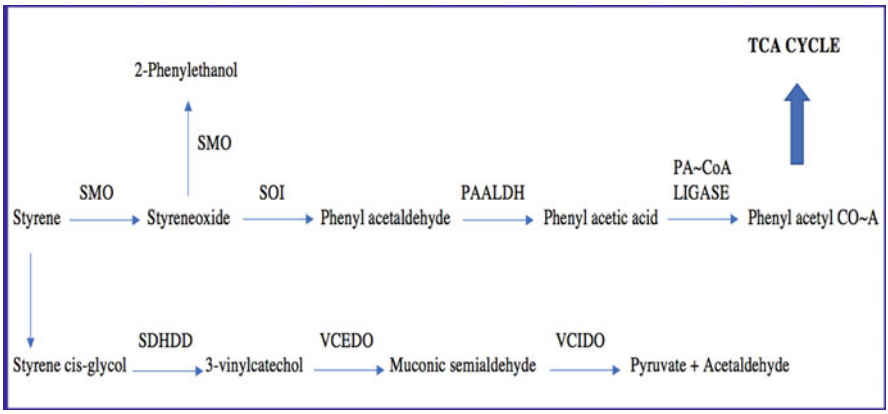

Fig. 13.5 Metabolic route for by bacteria for biodegradation of styrene (Adapted from: Mooney et al. 2006)

secreted by *Pseudomonas* sp. possess essential attributes in biodegradation of plastics. Various enzymes secreted by microorganisms such as PHA depolymerase are responsible for breakage of branch chains and the cyclic components present in various polymers. It has been reported that lipase secreted by *R. delemar*i degrade approximately 53% of the polyurethanes (ESPU) film-polyester type. Polyurethane esterase secreted by *Comamonas acidovorans* has been reported for their efficient biodegradation of ESPU having the constituent of polydiethylene adipate (Tokiwa et al. 2009). Different bacterial enzymes involved in biodegradation of styrene have been reported by Mooney et al. (2006) and have been documented in Fig. 13.5. Styrene biodegradation takes place by different sets of enzymatic reactions, and the process releases acetaldehyde, 2-phenylethanol, pyruvate, and 2-vinylmuconate as intermediate metabolites.

Side chain of styrene is oxidized to styrene oxide by styrene monooxygenase (SMO), phenyl acetaldehyde by styrene oxide isomerase (SOI), phenyl acetic acid by phenyl acetaldehyde dehydrogenase (PAALDH), and phenylacetyl-CoA by phenyl acetyl-CoA ligase (PA-CoA ligase). Phenyl acetyl-CoA obtained in the biodegradation process enters into the tricarboxylic acid (TCA) cycle that plays an important function in release of energy that has an integral role in metabolic and cellular pathways. 2-Phenylethanol is formed by the enzyme styrene monooxygenase (SMO) using the substrates styrene and phenyl acetaldehyde.

Styrene cis-glycol is formed by attack on aromatic ring of styrene by enzyme styrene 2,3-dioxygenase (SDO) with further formation of 3-vinylcatechol with aid of enzyme styrene 2,3-dihydrodiol dehydrogenase (SDHDD). 3-Vinylcatechol is further cleaved on ring to generate muconic semialdehyde by enzyme 2,3-vinylcatechol extradiol dioxygenase (VCEDD). Muconic semialdehyde is metabolized to pyruvate and acetaldehyde via 2,3-vinylcatechol intra-diol dioxygenase (VCIDO).

13.4 Toxicity of Polymers and Their Degraded Products

Polymers such as polypropylene and polyethylene are impervious to biodegradation process (Nicholson 2006). Degradation products formed after polymer degradation depend upon mechanism undertaken along with temperature and oxygen requirement during the process (Ravve 2000; La Mantia 2002). Polyacrylonitrile, nylons, and polyurethane commonly known as nitrogen-based plastics release hydrogen cyanide in thermal degradation process, whereas polymers rich in fluorene such as polytetrafluoroethylene and polyvinylidene fluoride release hydrogen fluoride following the mechanism of chain stripping (Lokensgard and Richardson 2004; Ravve 2000). Polymers proficient for depolymerization by chain scission into initial monomers comprises of polyoxymethylene, polytetrafluoroethylene, and polymethyl metacrylate. Fairly a high quantity of non-polymeric components (oligomers, lower molecular weight fragments, residual monomers, remains of catalysts, organic additives, and solvents) are present in polymers in bound or non-bound state. Most of them are highly hazardous to human health and are potent carcinogenic, mutagenic, and anti-reproductive metabolites. Being nonbiodegradable they have long persistence in the environment and are easily emitted out from polymeric product under usage in the biosphere (Crompton 2007).

13.5 Conclusions and Future Perspectives

As documented in several studies, plastic waste has become one of the major problems for environment, and in order to get rid of such a hazard, plastic wastes are usually put in landfills or burnt, which further causes serious threats to the ecosystem. Unfortunately, polymers like polystyrene, polyethylene, polyurethane, and polypropylene are not only durable but also the most robust plastics. With detrimental effects on plastic disposal, a holistic approach should be adopted for its clearance. This review discusses over the vital function of microorganisms in biodegradation of different plastic wastes. Enzymatic biodegradation of recalcitrant plastic waste serves as the most successful approach toward plastic disposal issues prevailing in the ecosystem. The complex polymeric structure of plastics is broken down into simple forms by different microorganisms employing biochemical transformation through enzymatic reactions. Biodegradation by microorganisms considerably reduces the release of byproducts that possess detrimental effect on the environment. The biocatalytically, plastic degrading enzymes in bacteria are esterases, lipases, proteases, and ureases, while in fungi, arsenal of ligninolytic enzymes is responsible for polymer degradation. Fungal endophytes have also shown promising results with respect to plastic biodegradation.

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Bioplastics: Fundamentals to Application

14

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Abstract

Plastics are major threat to the ecosystem, and growing consumer needs have contributed enormously to the widespread use and pollution of plastics. By 2050, it is estimated that around 12,500 metric tons of plastic waste will occupy landfills and the natural environment. Sustainable green technologies are therefore required to counteract the growing problem. Polyhydroxyalkanoates (PHAs) are biodegradable linear polyesters capable of replacing petrochemical plastics. Mostly functioning as sources of carbon and energy, PHAs can be derived either through microbial fermentation or through fungi and plants. Unlike conventional plastics, PHAs are biocompatible and non-toxic and have thermoplastic quality for use in the food, textile, medical and household industries. The present chapter focuses on the production, material properties and application of PHAs as functional bio-plastics and different strategies to alternate the plastic utilization. Microbes involved in different PHA productions in bioreactors, operational factors affecting bioplastic production and biochemical pathway associated with this have been illustrated. Further, challenges during scale-up studies for sustainable production and perspective have been discussed thoroughly.

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Keywords

Biodegradable plastic · Fermentation · Polyhydroxyalkanoates · Sustainability

14.1 Introduction

Since its discovery, plastics have been indispensable to mankind. Usage of plastics has been degrading the environment since decades because of their wide field of applications in every possible industry, even everyday household use (Fig. 14.1). Plastics have found variety of applications from packing to cellular mobiles and printers and from pharmaceuticals to automobiles. They are most useful synthetic polymers because of their structural durability in various shapes, sizes and strengths, high molecular weights and low reactivity and also durability. However, rapid urbanization and industrialization have caused huge volumes of these

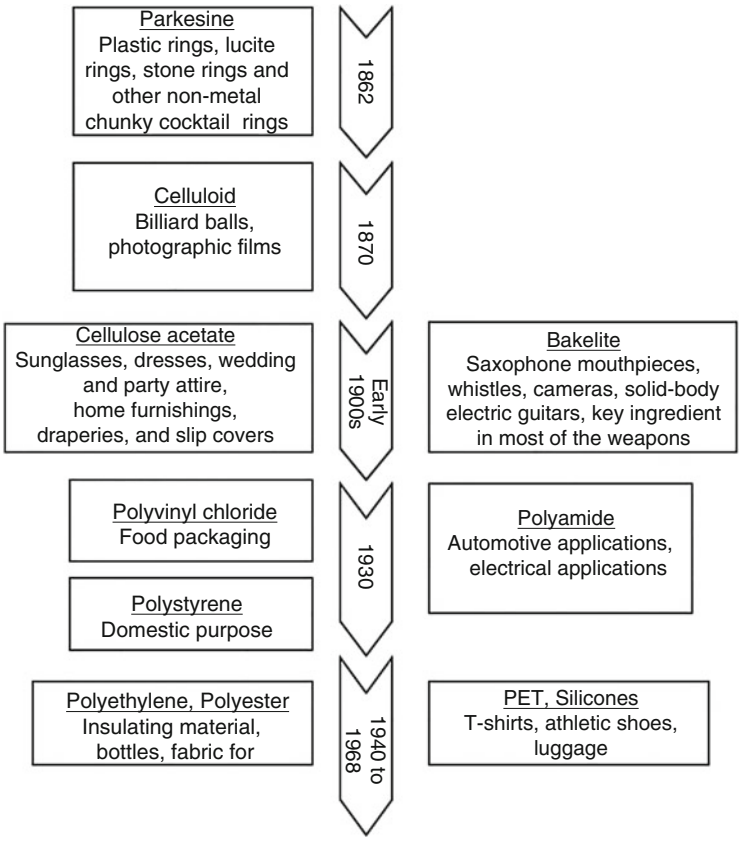

Fig. 14.1 A brief history of plastics and its application

non-degradable polymers to accumulate across planet. Consequently, rapid growth in plastic production to meet demands has irrevocably harmed the environment.

Plastic pollution due to improper plastic disposal, lack of regulations for plastic disposal and absence of recycling measures have created havoc for the ecosystem. The monomers involved in plastic production, such as ethylene, are obtained from oil, coal and natural gas and are not biodegradable. Consequently, they accumulate and remain in landfills for decades (more than 2000 years) instead of decomposing. Incineration, combustion and pyrolysis are viable options for permanent disposal, but it may lead to air pollution if carried out for large quantities of plastics (Barnes et al. 2009; DiGregorio 2009; Geyer et al. 2017). Pollution due to plastic in the marine environment is particularly rampant due to the dispersion of buoyant plastic fragments. These weathered fragments accumulate in gulfs, seas, oceans and coastlines gravely affecting the marine population. A recent research by Eriksen et al. suggested huge figures of more than 5250 billion plastic materials weighing 260,000 tons are afloat in water bodies (Eriksen et al. 2014).

Not only do plastics leave the environment fragile, but they also have severe consequences on human health. Phthalate esters, alkylphenols and other chemicals in plastics function as an endocrine-disrupting chemical, mimicking oestrogen and causing premature sexual development in young girls. Precocious puberty in young girls (onset of menstruation below the age of 8) is partially attributable to the use of phthalate containing plastics in the form of packaging materials, cosmetics, medical equipment, automobiles, toys and household items (Sanghavi 2006; Cesario and Hughes 2007). Plastics are now so prevalent that it has been proposed as a geological indicator of the Anthropocene epoch (Barnes et al. 2009; Zalasiewicz et al. 2016). Oil field depletion, growing costs of fossil fuels and crude oil and urbanization call for cost-competitive and sustainable alternatives. Development and production of biodegradable, green and sustainable plastic is the need of the hour. Over the last decade, a significant number of biodegradable biopolymers have been invented and produced from sustainable resources. Efforts have been made to use polymers like polyhydroxyalkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides and their copolymers or mixtures to satisfy the plastic requirements in different areas and sectors. Bio-based biodegradable polyhydroxyalkanoates (PHAs) commonly called bioplastic are gaining widespread attention as a potential alternative to polypropylene, polyethylene and polystyrene. A short journey on bioplastic production was depicted in Fig. 14.2.

Bioplastic polymer is either synthesized by microorganisms or derived by lignocellulosic biomass (Fig. 14.3).

Poly(3-hydroxybutyrate) [P (3HB)], the most common PHA, was first discovered by French scientist Lemoigne in 1926 in *Bacillus megaterium*, is a biodegradable linear polyester, having 3-, 4-, 5- and 6-hydroxy alkanoic acid monomeric units of D (−) configuration (Lee 1996a, b; Chee et al. 2010; Raza et al. 2018). PHAs are thermoplastics that accumulate as carbon- and energy-storing compound occupying up to 90% of cell volume. The basic structural skeleton of PHA is depicted in Fig. 14.4.

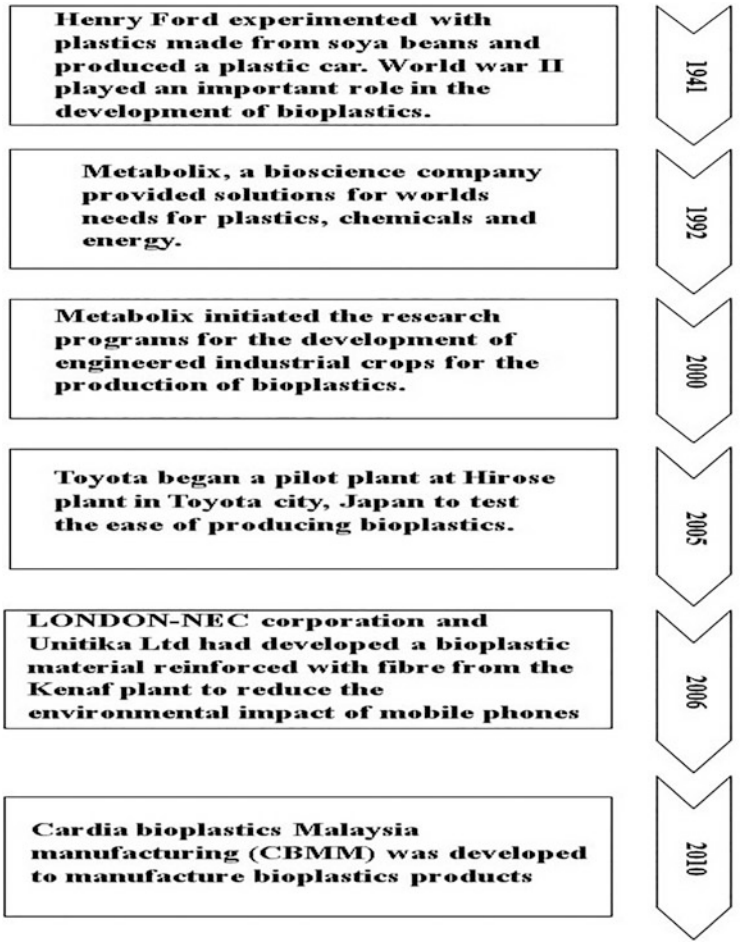

Fig.14.2 A chronological improvement on 'bioplastic production' research

14.2 PHA Inclusions

PHAs are produced intracellular in both Gram-positive and Gram-negative bacteria as a polymer (weights ranging from 2×10^5 to 3×10^5 D). The polymers accumulate as mobile amorphous or liquid granules/inclusions of different sizes (0.2–0.5 μm diameter) under nutrient-limited conditions of nitrogen, phosphorus, dissolved oxygen and magnesium (Steinbüchel and Füchtenbusch 1998; Saharan and Ankita 2012) and surplus of carbon (Anderson and Dawes 1990; Steinbüchel and Schlegel 1991; Lee 1996a, b; Steinbüchel and Füchtenbusch 1998; Suriyamongkol et al. 2007). Electronic microphotographs depict these inclusions as electronucleant and light-refracting bodies. These granules are encapsulated by a phospholipid

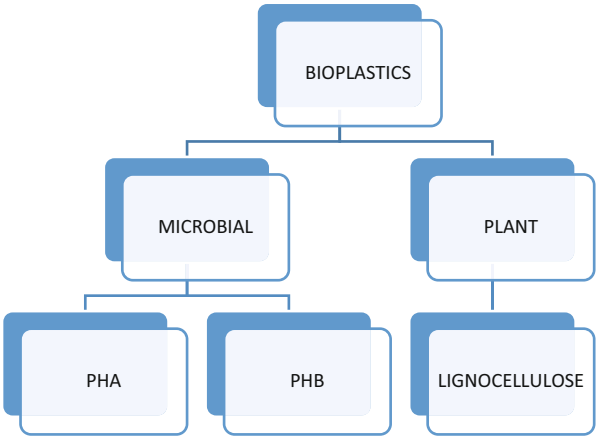

Fig. 14.3 Different biocatalysts for of bioplastic production

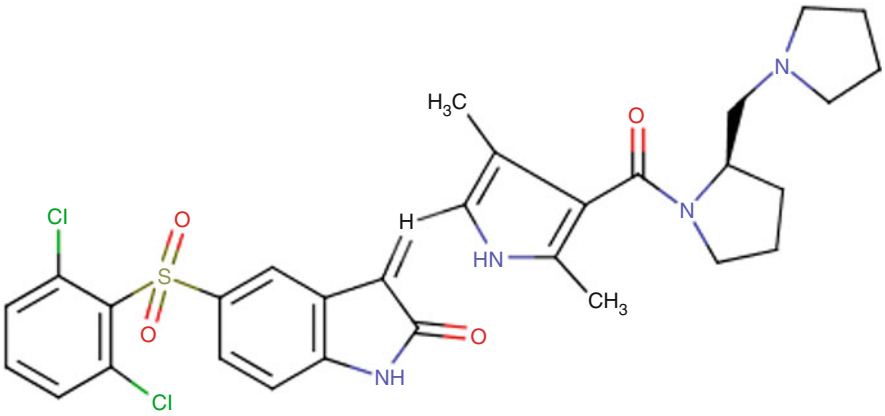

Fig. 14.4 Basic structure of PHA

monolayer containing intracellular PHA depolymerase, polymerase and phasing proteins (Barnard and Sanders 1989; Sudesh et al. 2000).

14.2.1 Polyhydroxyalkanoate Synthase

The polymerizing enzyme, PHA synthase (PhaC), is responsible for the type of PHA synthesized by the microorganism. The enzyme can be categorized into three types based on substrate specificity, subunits and structure.

Class 1: Specific towards scl-HA monomer having a single subunit (60–70 kDa). Identified in *Ralstonia eutropha*.
Class 2: Specific towards mcl-HA monomer having a single subunit (60–70 kDa). Identified in *Pseudomonas oleovorans.*
Class 3: Specific towards scl-HA, also polymerizes scl- and mcl-monomers (Steinbüchel and Schlegel 1991; Nishikawa et al. 2002; Suriyamongkol et al. 2007)

These synthases have two subunits of 40 kDa each and have been identified in *Chromatium vinosum*, *Thiocystis violacea* and *Thiocapsa pfennigii* (Liebergesell and Steinbüchel 1992, 1993; Liebergesell et al. 1993; Poirier et al. 1995; Hein et al. 1998; Sudesh et al. 2000).

14.2.2 PHA Depolymerase

Intracellular PHA depolymerase (PhaZ) helps in depolymerization of reserved carbon. On the other hand, extracellular depolymerase, secreted by several microorganisms, is used for degradation of crystalline PHA in the surroundings (Chowdhury 1963; Delafield et al. 1965; Lee and Choi 1999).

14.2.3 Phasins (PhaP)

Present on the surface of the phospholipid layer, phasin proteins are involved in stabilization and protection of PHA inclusions inside the cell cytosol. Microscopic methods, namely, SEM and TEM (scanning and transmission electron microscopy) and AFM (atomic force microscopy) are used for PHA granule studies. These studies give us an estimate of the granule size and number, granule structure, molecular weights and other physiochemical properties such as crystal structure and melting temperature. However, these are variable parameters and are dependent on the producing microorganism, available substrate and carbon source. Techniques such as size exclusion chromatography and sedimentation analysis are employed for determining molecular weights, whilst gas chromatography, mass spectroscopy and NMR are used for monomer composition analysis (Sudesh et al. 2000). Sudan black B or Nile blue A staining could be performed for easy identification of native PHA inclusions (Burdon 1946; Ostle and Holt 1982; Sudesh et al. 2000).

14.3 Characterization of PHAs

PHAs can be classified based on molecular weight or chain length. High-molecular-weight PHAs are produced as granular storage material in archaebacterial, Gram-positive and Gram-negative bacteria under unfavourable growth environments. Short-chain-length (scl) PHAs consisting of 3HB,3-hydroxy valerate (3 HV) and

4HB monomers are found in *Cupriavidus necator* and contain up to 5C atoms in their side chains. Medium-chain-length (mcl) PHAs consisting of 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) monomers accumulate in several *Pseudomonas* sp. and consist 6–14 carbon chain polymers. Lastly, long-chain-length (lcl) polymers having greater than 14 carbon atoms in their side chains are found in organisms such as *Aureispira marina*. Structurally, these chains can be saturated or unsaturated and straight or branched with aliphatic or aromatic side groups. The difference in chain length arises due to the substrate specificity of PHA synthases (Kunioka et al. 1989; Doi 1990; Saito et al. 1996; Witholt and Kessler 1999; Chee et al. 2010).

PHAs typically constitute either mcl monomers or long-chain-length monomers. PHA monomers have been identified to be straight, branched, unbranched, saturated, unsaturated and aromatic. Presence of functional groups as such halogens, carboxyl, hydroxyl, epoxy, phenoxy, cyanophenoxy, thiophenoxy and methyl ester groups allows chemical alternation. The length of the side chain and the functional groups often affect the degradability of the bioplastic (Eggink et al. 1995, Kessler et al. 2001, Kim and Lenz 2001, Zinn et al. 2001).

14.4 Biosynthesis of PHAs

PHA production is majorly dominated by bacterial species belonging to family *Halobactericeae* (Anderson and Dawes 1990; Steinbüchel and Valentin 1995; Braunegg et al. 1998; Madison and Huisman 1999; Zinn et al. 2001; Berlanga et al. 2006; Ciesielski et al. 2006; Suriyamongkol et al. 2007). Several biosynthetic pathways for the production of PHAs are identified in bacteria. The most significant pathways include the ones in *Ralstonia eutropha*, *Rhodospirillum rubrum* and *Pseudomonas* sp.

The biochemical road map for the synthesis of PHA in *Ralstonia eutropha* is a three-step condensation, reduction and polymerization reaction (Fig. 14.5). Enzyme β-ketothiolase condenses two molecules acetyl-CoA to acetoacetyl CoA. The acetoacetyl CoA is stereoselectively reduced by the acetoacetyl-CoA reductase to

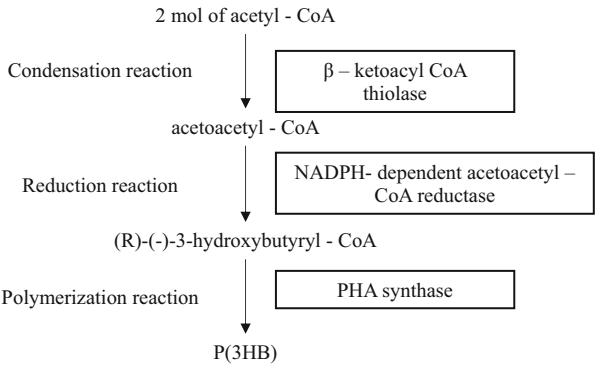

Fig. 14.5 Biosynthetic pathway for the formation of PHA in *R. eutropha*

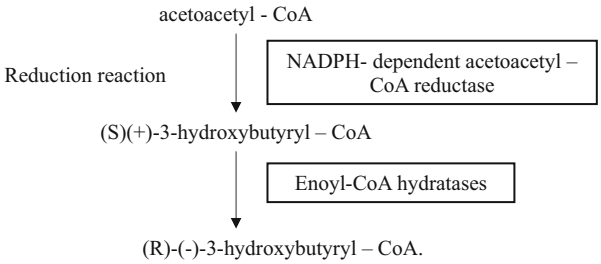

Fig. 14.6 Biosynthetic pathway for the production of PHA in *Rhodospirillum rubrum*

(R)-(−)-3-hydroxybutyryl-CoA. Finally, PHA synthase catalyses polymerization of the (R)-(−)-3-hydroxybutyryl-CoA molecules to P(3HB) via an ester linkage (Moskowitz and Merrick 1969; Anderson and Dawes 1990; Doi 1990; Lee and Choi 1999).

The pathway for the production of PHA in *Rhodospirillum rubrum* is a five-step process similar to *R. eutropha* (Fig. 14.6). The difference lies in that acetoacetyl-CoA first condenses to (S)(+)-3-hydroxybutyryl-CoA and subsequently to (R)-(−)-3-hydroxybutyryl-CoA. Unlike in *R. eutropha*, mcl-PHA biosynthesis is focused in *Pseudomonas oleovorans* and other *Pseudomonas* sp. belonging to rRNA homology group I. The pathway utilizes intermediates such as enoyl-CoA, 3-ketoacyl-CoA and (S)-3-hydroxy acyl-CoA, generated from degradation of fatty acids (Langenbach et al. 1997; Qi et al. 1997; Ren et al. 2000; Park et al. 2002; Suriyamongkol et al. 2007). Another pathway that uses acetyl-CoA to synthesize mcl-PHA polymers is utilized by *P. aeruginosa* and most organisms belonging to rRNA homology group I except *P. oleovorans* (Suzuki et al. 1986; Haywood et al. 1990; Huijberts et al. 1992; Lee 1996a, b).

14.4.1 Molecular Understanding of PHA Synthesis

Three main steps dictate the synthesis of mcl-PHA: chain elongation of fatty acids, β-oxidation of fatty or alkanoic acids and de novo fatty acid synthesis. The key reaction to mcl-PHA biosynthesis is the transesterification reaction catalysed by the mcl-PHA polymerase that links monomer units. The mcl-PHA genes are regulated by the *phaC1ZC2D* operon which contains polymerases phaC1 and phaC2 and depolymerases Pha2 and Pha1 protein. The operon expression is controlled by promoter sequences upstream of phaC. *pha*F1 located downstream of the operon encodes the PhaF and PhaI phasins and is involved in the stabilization, regulation and formation of PHA granules (Lageveen et al. 1988).

14.5 Production of PHA

PHA production utilizes renewable resources as raw materials, CO_2 and sunlight for production by plants and glucose for production via fermentation.

14.5.1 PHA Production in Microbes

More than 300 species of microorganisms over 90 genera are known to synthesize and accumulate PHA in their cytoplasm (Zinn et al. 2001; Kim et al. 2007; Muhammadi et al. 2015; Raza et al. 2018) under oxygen-sufficient and oxygen-deficit conditions. The selection of bacteria for production depends on commonly involved microorganisms in bioplastic production including *Cupriavidus necator*, *Aeromonas hydrophila*, *Rhodopseudomonas palustris*, *Bacillus* sp., *Pseudomonas* sp., *Methylobacterium* sp. and *Escherichia coli.* The microorganisms producing PHAs can be characterized into two groups based on nutrient requirements. Bacteria such as *Pseudomonas* sp., *Bacillus* sp., *Methylobacterium* sp., *Ralstonia eutropha* and C. *necator* cannot synthesize PHA in their growth phase and need nutrient-deficient environment of N_2, P, O_2 or Mg and surplus of C, whereas organisms such as *Alcaligenes eutrophus*, *Alcaligenes latus* and recombinant *E. coli* synthesize PHA in their growth phase in non-nutrient-limiting conditions (Lee 1996a, b; Khanna and Srivastava 2005; Chee et al. 2010; Raza et al. 2018). Both wild-type and recombinant bacteria are used for the production of scl and mcl-PHAs. Owing to its high cell density (100–200 g/L) and PHA content (75–80%), *Ralstonia eutropha* is the widely exploited wild strain for scl-PHA production. *Aeromonas hydrophila* and *Pseudomonas oleovorans* are employed for mcl-PHA production.

14.5.2 Fermentation Process

Batch and fed-batch fermentation techniques have been widely used for PHA production. Batch fermentation is favourable due to low operational cost, easy screening and growth analysis of the compound. However, the yield is affected due to degradation of accumulated PHAs by bacterial enzymes. Comparatively, fed-batch cultivation has greater PHA productivity, but yield decreases in the presence of limited nitrogen levels. PHAs are usually produced fermentatively as a two-stage fed-batch method. The initial growth stage is nutritionally enhanced to yield maximum biomass, followed by a nitrogen-depleted stage. Single fed-batch fermentation that is nitrogen restricted results in low polymer quantities because of insufficient accumulation of biomass (Doi 1990; Katırcıoğlu et al. 2003; Verlinden et al. 2007). To obtain high productivity without losses, both techniques can be combined to produce PHA via a two-stage process. The primary step involves PHA accumulation under batch conditions by the rapid growth of microorganism until a desirable amount of biomass is produced. The second step is shifting fermentation conditions to a fed-batch mode in the late exponential phase, maintaining limited conditions of nitrogen and continuous flow of carbon. Culture conditions maintained at an 18–20 °C temperature and pH 7 with constant aeration can boost PHA accumulation (Zinn et al. 2001; Ibrahim and Steinbüchel 2009; Saharan and Ankita 2012; Amache et al. 2013). Controlled fermentation method, the chemostat, is a technique wherein the culture broth is continually replenished with a fresh non-contaminated growth medium. Jung et al. obtained 63% (w/w) mcl-PHA of

cell dry weight through chemostat fermentation. Although this cultivation method provides high productivity, large-scale applications have not been achieved (Jung et al. 2001; Zinn et al. 2001). Zinn et al. (2001) reported PHA production using *P. oleovorans* that accumulated PHA when supplied simultaneously with growth-limiting n-octane and ammonium as carbon and nitrogen substrate, respectively. The dual substrate-limited growth is also a beneficial method to produce PHA from the toxic carbon source (De Smet et al. 1983; Zinn et al. 2001). Fermentation efficiency can be improved by using mixed cultures. *Cupriavidus necator* cannot break down sugars, whey or molasses. A mixed culture of lactic acid producing microbes that converts substrates into the utilizable form along with can be used (Patnaik 2005; Verlinden et al. 2007).

14.5.3 PHA Production through Recombinant DNA Technology

Production of PHA in natural isolates is a tedious affair. High granule purification costs, long production time and the high cost of media are contributing factors. Additionally, the granules are hard to lyse, and the natural pathways often contain PHA-degrading enzymes. Recombinant technology is an attractive option. *Pha* CAB operon of *Ralstonia eutropha* cloned in *E. coli* and *Pseudomonas oleovorans* yielded PHA around 70–90% under fed-batch conditions. Recombinant organisms possess a high growth rate, high cell density and the ability to grow in cheap substrates. Such manipulations lead to efficient and cost-effective production and increased yield in cultures.

14.5.4 PHA in Plants

Bulk production of PHA in bacterial and yeast system is economically draining. Plants utilize available CO_2, H_2O and sunlight to accumulate PHAs in their cytosol, plastids and peroxisomes. C4 pathway utilizing plants yields high biomass on marginal land by effectively using water, nitrogen and energy. It is estimated that PHAs can be produced in plants at 0.20–0.50 US$/kg at 20–40% dry wt. It is cost-competitive and agronomically attractive. C4 plants such as sugarcane, maize, sorghum and switchgrass are being engineered for the production of PHA as a co-product using PHA synthase.

Enzyme β-ketothiolase has been discovered in the cytoplasm of some greater crops, which implies that the plant requires only reductase and PHA synthase to synthesize PHA. An oil plant designed harbouring the *A. eutrophus* PHA genes collected 0.2–0.5 μm of PHB granules in the cellular organelle such as nucleus, vacuole and cytoplasm. The amount of PHB was 100 μg/g fresh weight; however, crop development was impaired due to substrate depletion. Subsequently, genetic manipulations were performed in the PHA genes to divert from endogenous metabolic pathways and were inserted in plastids of *Arabidopsis thaliana*. The new

hybrid plant accumulated about 10 mg/g fresh weight, about 14% of the dry weight of PHB (Poirier et al. 1995; Lee 1996a, b; Ojumu et al. 2004).

In a plastid engineering-synthetic biology-coupled approach, genes *phaA* and *phaB* (from *Acinetobacter* sp.) and *phaC* (*Bacillus megaterium*) encoding thiolase, synthase and reductase were introduced in tobacco plastids through particle bombardment method. Transplastomic tobacco obtained in this study successfully produced PHB up to 17.3% and 8.8% dry weight in leaf tissue and biomass of the total tobacco plant, respectively. Through a similar synthetic biology and genetic engineering approach, it has been successfully demonstrated that an entire operon for PHB (phaCAB) from *Ralstonia eutropha* when placed in SE100 plant vector avoids the rigorous technical procedures for production of PHB in tomato plants. Similarly, through particle bombardment of a gene construct (involving bacterial genes for PHB production, viz. *phaC*, *phaB* and *bktB*) in embryonic calli of *Elaeis guineensis* Jacq. (oil palm), synthesis of PHB was successfully achieved. Several recent reports can be reviewed which signify the advantages and utilities of plants as bio-factories for the production of PHB.

14.5.5 PHA Production from Waste Substrates

Accumulation of PHA happens under aerobic conditions, leading to increased intracellular loss of the carbon substrate through respiration. Less than 50% of the carbon source is targeted for PHA formation. Additionally, high manufacturing costs make PHAs significantly more costly than synthetic materials (Steinbüchel and Füchtenbusch 1998). Manufacturing from locally accessible and renewable sources of carbon would be more economical (Ojumu et al. 2004). Using waste by-products such as starch and lipids that have been modified as substrates for PHA synthesis is widely researched upon for a cost-efficient approach.

14.5.5.1 Production from Plant Waste

Luis et al. utilized cellulosic waste from tequila bagasse for the production of bioplastic under nitrogen-deficient culture conditions using *Saccharophagus degradans*. A minimal media supplemented with glucose, cellobiose, apical and bagasse confirmed the production of PHA (Alva Munoz and Riley 2008).

14.5.5.2 Production from Biological Waste

Volatile fatty acids (VFAs) are metabolic products derived by hydrolysation of compounds present in industrial effluents and damaged fruits and vegetables. VFAs are utilized anaerobically by microorganisms such as *Bacillus megaterium*, *Pseudomonas oleovorans* and *A. beijerinckii* as substrates for PHA production.

14.5.5.3 Production from Activated Sludge

Satoh et al. (1998) demonstrated the use of activated sludge as a starting material for PHA production. Up to 62% of PHA accumulation was obtained by maintaining microanerophilic-aerobic conditions. Additionally, activated sludge PHA production is cost-effective as production conditions are not as stringent as traditional fermentation techniques (Satoh et al. 1998).

14.5.5.4 Production from Wastewater

Different types of waste were used for bioplastic production. This has been listed in Table 14.1. Yan et al. reported the use of paper and pulp wastewater for production of PHA and obtained a yield of 43% (Yan et al. 2008). Ryu et al. using swine wastewater as the substrate obtained a PHA yield of 58%.

14.6 Recovery of PHAs

Product recovery is the next crucial step after production that needs to be handled for profitable production of PHB. The recovery of product is achieved with the help of a wide array of technique involving solvent extraction, enzymic digestion and dispersion. Powerful techniques such as centrifugation and filtration are used with a particular solvent for product extraction and isolation (Fig. 14.7).

Recovery of PHAs can be a costly affair. The most widely used recovery method is solvent extraction. Although the yield and purity obtained through this method are advantageous, the excessive use of solvents is not environmentally friendly. Recent alternatives include dispersion process, selective cell mass dissolution and enzymatic recovery using trypsin, chymotrypsin, rennin and papain (De Koning and Witholt 1997; Chee et al. 2010). Different other methods are also employed to recover PHA in a cost-effective manner (Table 14.2).

14.7 Biodegradation of PHA

The uniqueness and major interest in bioplastics lie in degradation part. Unlike petro-based plastics, the PHA can be digested using microbes. This attribute of PHA eliminates the continuous piling of dumping bioplastic.

Polyhydroxyalkanoates such as PHB, P(HB-HV) and others function as a major energy and carbon source for microorganisms, which colonize on the polymer surface to produce enzymes that would help facilitate the degradation of the polymer into its monomeric constituents such as P(HB-HV) degrades to give HB and HV. These units are then utilized by the cell for biomass production (Luzier 1992; Mergaert et al. 1993, 1994; Poirier et al. 1995; Lee 1996a, b; Ojumu et al. 2004). PHA degradation is a two-step process. Firstly, depolymerases break down PHA polymers into monomers, dimers or oligomers (Nakayama et al. 1985; Jendrossek et al. 1993; Schirmer et al. 1993; Lee and Choi 1999). These monomers are taken up by the cell and catabolized under suitable aerobic and anaerobic conditions, producing CO_2, water and biomass in the presence of oxygen and carbon dioxide and methane in the absence of oxygen. Microorganisms such as *Cupriavidus necator*, *Alcagenes latus* and *Aeromonas hydrophila* are capable of decomposing PHAs into H_2O and CO_2. PHAs are not only compostable but can also be depolymerized into R-(−)-configured hydroxy acids exhibiting antiviral and antibacterial properties.

Table 14.1 Bioplastic production from various wastewater resources

S. no.	Waste category	Substrates used	Microorganisms used	References
1.	Lignocellulosic waste	Forest biomass	• *Burkholderia cepacia* P(3HB)	Young et al. (1994), Keenan et al. (2006), Koller et al. (2010)
		Rice straw Corn straw Bagasse Poplar wood	• *P. cepacia* ATCC 17759 P(3HB)	
2.	Diary waste	Cheese	• *Hydrogenophaga pseudoflava* • *Haloferax mediterranei*	Yellore and Desai (1998), Povolo and Casella (2003), Koller et al. (2007a, 2007b, 2007a, b)
		Cheese whey	• *Methylobacterium* sp. ZP24	
3.	Biodiesel waste	Glycerol liquid phase (GLP)	• *H. mediterranei* • *Methylobacterium rhodesianum* MB126 • *Ralstonia eutropha* DSM 11348 • *Pseudomonas oleovorans* NRRL B-14682 for P(3HB) • *Pseudomonas corrugata* 388 mcl-PHA	Bormann and Roth (1999), Ashby et al. (2004, 2005), Koller et al. (2005, 2010)
4.	Lipid waste	Waste cooking oil	• *Pseudomonas aeruginosa* 42A2	Cromwick et al. (1996), Füchtenbusch et al. (2000), Fernández et al. (2005), Koller et al. (2010)
		Residual oils	• *Pseudomonas oleovorans* • *C. necator* H16	
		Tallow	• *Pseudomonas resinovorans*	
5.	Slaughterhouse waste	Meat and bone meal	• *H. mediterranei*	Koller et al. (2005, 2010)
6.	Waste stream	Swine waste liquor	• *A. vinelandii*	Chou et al. (1997), Lee and Choi (1999)
		Alcohol distillery waste	• *Actinobacillus* sp.	Son et al. (1996)

(continued)

Table 14.1 (continued)

S. no.	Waste category	Substrates used	Microorganisms used	References
7.	Starch	Waste potato starch	• *Ralstonia eutropha* NCIMB 11599	Rusendi and Sheppard (1995), Haas et al. (2008), Koller et al. (2010)
		Barley malt	• *Alcaligenes eutrophus*	
8.	Plant waste	Palm oil, crude palm oil, palm olein	• *Cupriavidus necator* H16	Füchtenbusch et al. (2000); Lee et al. (2008) Gonzalez-Lopez et al. (1996), Majid et al. (1999), Ribera et al. (2001), Pozo et al. (2002), Koller et al. (2010)
		Crude palm oil	• *Erwinia* sp. USMI-20	
		Alpechin from olive oil	• *Azotobacter chroococcum* H23 • *Pseudomonas putida* KT2442	

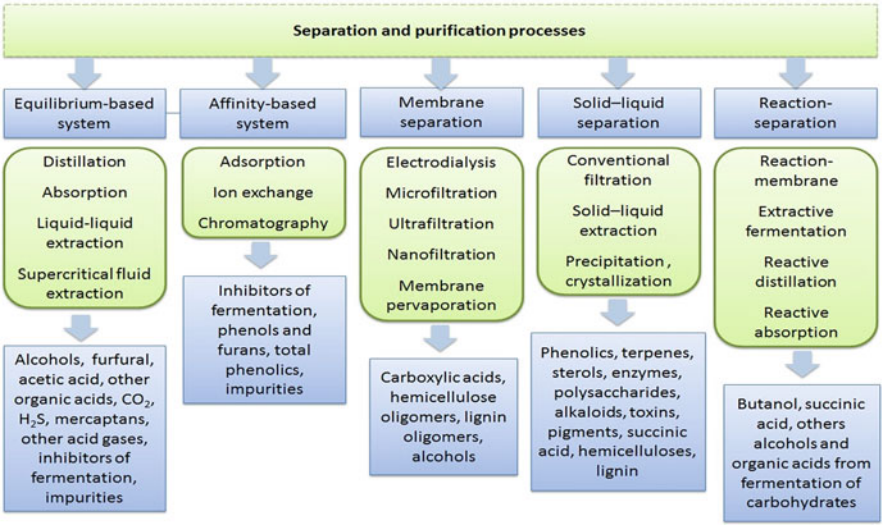

Fig. 14.7 Varieties of methods for the efficient recovery of PHAs

Degradation of PHAs is influenced by the type of PHA depolymerase, monomer composition and environmental conditions such as temperature, soil, water, salinity and other properties. Compostable temperature can range anywhere from 6 to 70 °C. Time duration also varies greatly. Eighty-five degradation over 7 weeks was achieved with 60 °C and 55% moisture conditions. In aquatic conditions, it can take longer than 250 days at temperature 5 °C. Additionally, UV radiation can accelerate degradation.

Table 14.2 Other methods for recovery PHA

S. no	Recovery method	Technique/ materials used	Advantages	Yield obtained	References
1.	Solvent extraction	Chloroform, acetone, ethylene carbonate and 1,2 propylene carbonate, methylene chloride used for extraction	1. High purity 2. Removes endotoxins 3. No polymer degradation	High yield	Furrer et al. (2007), Jacquel et al. (2008), Raza et al. (2018)
2.	Supercritical fluid extraction	Supercritical CO_2, methanol, ammonia	1. Low toxicity 2. Low cost 3. High purity	1. 89% PHA recovery from *Ralstonia eutropha* 2. 42% mcl-PHA recovery from *Pseudomonas resinovorans*	Hampson and Ashby (1999), Hejazi et al. (2003), Khosravi-Darani et al. (2003), Kunasundari and Sudesh (2011), Raza et al. (2018)
3.	Floatation method	Solvent extraction with chloroform followed by self- flotation	Environment-friendly	85% PHA recovery	Ibrahim and Steinbüchel (2009), Raza et al. (2018)
4.	Chemical digestion	Digestion with triton-X, SDS, betaine with sodium hypochlorite to release PHAs	High purity	5.6 g/l recovery of PHBs	Jacquel et al. (2008), Sayyed et al. (2009), Koller (2016), Raza et al. (2018)
5.	Aqueous two-phase extraction	Extraction using water and non-volatile phases (polyethylene glycol and potassium phosphate)	Environment-friendly	51% PHA recovery	Kepka et al. (2003), Raza et al. (2018)

14.8 Applications of PHAs

Mcl-PHAs are highly elastic and flexible and possess low levels of crystallinity, high melting point, and increased resistance towards brittleness compared to PHB and scl-PHA, making it an ideal raw material for application in various fields. Owing to

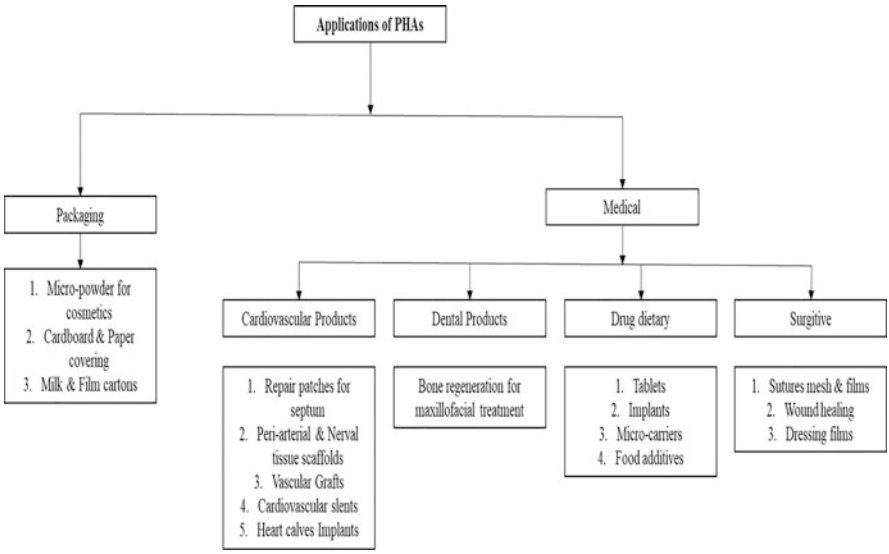

Fig. 14.8 Different application of PHAs

structural differences in their monomer units, PHAs have versatile properties. They are biodegradable, piezoelectric, insoluble in water, immune to hydrolytic damage and unaffected by UV radiation.

These properties make them more selective than polypropylene. It also has a wide range of application due to their novel features (Fig. 14.8).

It is used as osteo-synthetic substance for stimulating bone development because of their piezoelectric property. The medicinal uses are still limited due to slow decay rate and higher stability in tissues.

14.9 Conclusions

Bioplastics are the emerging solution to the no degradable plastics in our everyday use. These bioplastics are produced from all the natural biodegradable plant sources and also cost-efficient resource. The current major advantage of bioplastic products is in reducing the permanent litter and creating reusable products. At current stage, limiting factors for successful scale-up of this technology are the slow production kinetics and high production cost. In spite of several advantages, bioplastic production-related research is mostly confined at a small scale. Further, efficient engineered strain needs to be developed in order to increase the maximum productivity.

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Microbial Electrochemical Dye Degradation: Present State of Art

15

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Abstract

Dyes are commonly utilised by many industries, for instance, pharmaceuticals, cosmetics, leather and textiles. Out of which, the textile-based industries produce most of the wastewater which is a major source for pollution in the world, as it contains a mixture of dyes, additives and chemicals which were added during textile manufacturing. These dyes are detrimental to the environment as most of the dyes are recalcitrant. Without treatment of dye-containing wastewater, dyes may accumulate in the fishes and other aquatic flora and fauna. It further causes carcinogenic or mutagenic conditions. It can also cause skin irritation, allergies or dermatitis. Microbial fuel cell (MFC) is a promising microbial-mediated electrochemical wastewater treatment tool where electroactive bacteria can be utilised for degrading the dyes with simultaneous electricity generation. Dyes could be removed both in anodic and cathodic chambers using the oxidation and reduction capabilities of bacteria, respectively. This chapter has highlighted principle of MFCs for dye biodegradation, microbes associated with dye degradation along with catabolic pathway responsible for dye degradation. Also, different case studies for cationic, anionic and non-ionic dyes with MFCs, factors affecting dye degradation and challenges in MFC operation are taken into account.

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Furthermore, it also discusses the materials used in MFCs, the different types of dyes, their structure and effects along with the pros and cons of the dye degradation techniques of MFC compared to other existing processes.

Keywords

Dyes · Carcinogenic · Microbial fuel cell · Biodegradation · Bioelectricity · Exoelectrogenic bacteria

Abbreviations

AY	Alizarin Yellow R
BOD	Biological oxygen demand
CA	Chronoamperometry
COD	Chemical oxygen demand
CP	Chronopotentiometry
CV	Cyclic voltammetry
EIS	Electrochemical impedance spectroscopy
FAD	Flavin adenine dinucleotide
GAC	Granular activated carbon-bio cathodes
GDL	Gas diffusion layer
LSV	Linear sweep voltammetry
MFC	Microbial fuel cells
MWCNT	Multi-walled carbon nanotubes
NAD	Nicotinamide adenine dinucleotide
OLR	Organic loading rate
PEM	Proton exchange membrane
TDS	Total dissolved solids
TOC	Total organic carbon

15.1 Introduction

Dyes can be labelled as chemical stuff that binds to material and gives colour to that material. Different materials are coloured using dyes, materials like food, leather, plastic, paper, fur, hair, textile fibre, cosmetics, etc. (Benkhaya et al. 2017). In the textile industry, dyes used generally consist of a group of atoms called chromophores, which consists of diverse functional groups such as carbonyl, azo, nitro, etc. Azo dyes contain at least one azo bond, i.e., nitrogen-nitrogen double bond (N=N); however, there are different structures relying on the number of azo bonds (N=N) present in them, for example, monoazo dyes consists of single azo bond, diazo dyes have two azo bonds and, likewise, triazo dyes have three azo bonds. Also, the azo groups are by and large bound to naphthalene or benzene rings. Structures of some of the common dyes used in the industries are depicted in Figs. 15.1–15.5.

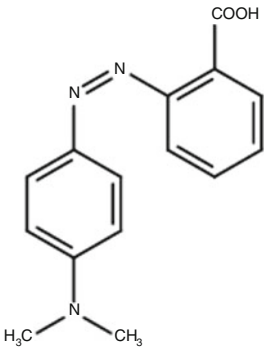

Fig. 15.1 Methyl red-monoazo dye. Depicts some examples of some azo dyes structures (adapted from: "Microbial degradation of Azo Dyes: A review", Sudha et al. 2014)

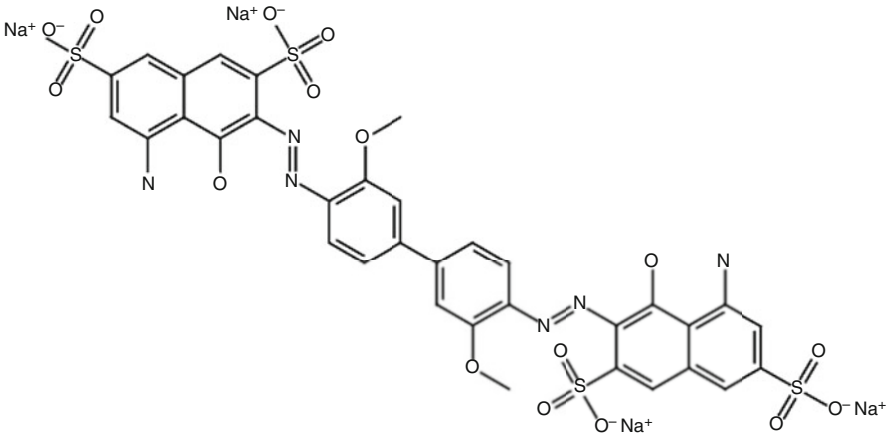

Fig. 15.2 Direct blue 15-diazo dye. Depicts some examples of some azo dyes structures (adapted from: "Microbial degradation of Azo Dyes: A review", Sudha et al. 2014)

The textile industry adds to significant contribution in total industrial production of countries like India, China, the UK, etc. A major share of dyes is utilised by the textile industry. The textile industry amongst the various industries is considered to have one of the most polluting wastewater effluents in the world, with regard to composition, volume and large quantities of dyes that are used for colouring fabrics. Approximately 10–15% of the dyes are released in the liquid waste during the dyeing procedures (Baban et al. 2003). A large portion of the dyes, about 200,000 tons is adrift to effluents in the textile industry every year, and 60–70% of dyes produced in the world are accounted for by azo dyes (Uddin et al. 2012). The concentrations at which the dyes and pigments are discharged from industries to the water bodies are harmful and toxic to humans and aquatic life. Further, the dyes used to raise the level of the biological oxygen demand (BOD), alter the pH, increase chemical oxygen demand (COD) and immensely reduce water quality.

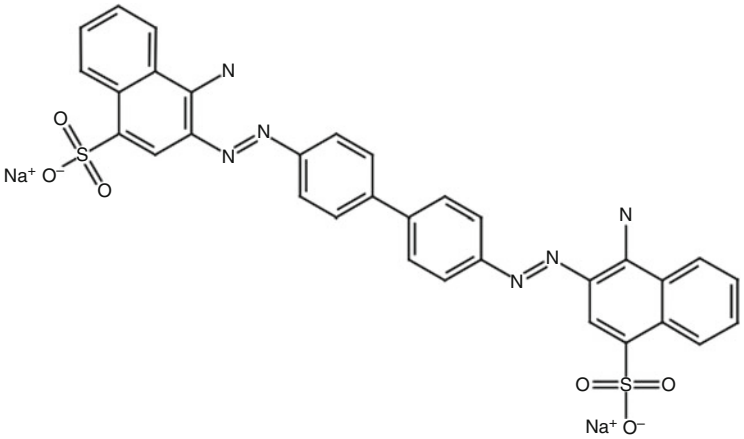

Fig. 15.3 Congo red dye. Depicts some examples of some azo dyes structures (adapted from: "Microbial degradation of Azo Dyes: A review", Sudha et al. 2014)

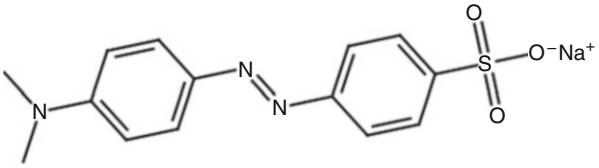

Fig. 15.4 Methyl orange dye. Depicts some examples of some azo dyes structures (adapted from: "Microbial degradation of Azo Dyes: A review", Sudha et al. 2014)

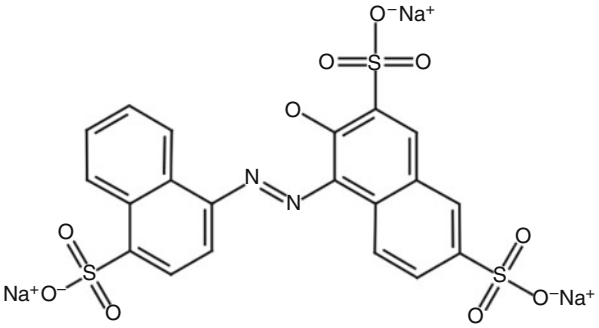

Fig. 15.5 Amaranth dye. Depicts some examples of some azo dyes structures (adapted from: "Microbial degradation of Azo Dyes: A review", Sudha et al. 2014)

The natural degradation of such azo dyes is very hard due to their high chemical stability (Shaikh et al. 2016). Even if the dyes are degraded through different processes, such as chemical methods like coagulation, flocculation, oxidation and

reduction, physical methods employing reverse osmosis, precipitation and adsorption and biological processes such as aerobic and anaerobic treatments, the degradation products are often mutagenic or carcinogenic (Méndez-Paz et al. 2005). Thus, the development of such a technology that can tackle these difficulties is much required.

Recent research proved microbial fuel cell (MFC) as a potential tool for degrading inorganics and organics into useful electrical energy and facilitate a reduction in COD, TDS and BOD (Jung and Pandit 2019). This is made possible with the aid of exoelectrogen or electroactive bacteria as catalysts for the electricity production and concurrent decolourization of the wastewater containing azo dyes (Solanki et al. 2013). Furthermore, MFC treatment generates 50–90% fewer solids to be disposed of. A typical MFC comprised of three components – electrodes, membranes and substrate. The electrodes (mostly anode) showed specific characteristics such as high conductivity and effective electron transport ability between the bacteria and electrodes (Wei et al. 2011). Furthermore, the absence of such a membrane causes an increase in oxygen and substrate diffusion which in turn reduces the bio-catalytic activity and Coulombic efficiency of the anode microorganisms (Hou et al. 2011). The third important factor is a substrate which supports all the biological activities in the MFCs. MFCs are operated using simple organic substances like acetate, glucose to complex substrates in the form of industrial wastewater and synthetic wastewater, lignocellulosic biomass, etc. (Mathuriya 2014). Different case studies for treating cationic, anionic and non-ionic dyes with the help of MFCs are seen here. Furthermore, the chapter also sheds some light upon the factors affecting dye degradation and challenges in MFC operation and also the materials used in MFCs, the different types of dyes, their structure and effects along with the advantages and disadvantages of the dye degradation methods.

15.2 Problems Associated with Azo Dye Disposing

Dyes are mostly of two types – natural and synthetic. The things found in nature are actually the sources of natural dyes, and hence these natural dyes are easily biodegradable, causing no harm to the surroundings, whereas the synthetic dyes are made by man, i.e., artificial, thus causing problems to the environment and society. Still synthetic dyes are utilised a lot by many industries like pharmaceuticals, cosmetics, textiles and leather. Out of which, the textile industries generate most of the wastewater which leads to pollution in the world, as it contains a mixture of dyes, additives, and chemicals which were added during textile manufacturing. When the clothes are coloured with the dyes, a high percentage of dyes do not attach to the fabric well leading to its disposal in wastewater. Around 70% of these dyes are azo dyes with azo bond (-N=N-) and carbon groups (organic). These azo dyes are more popular because they dye cloth at 60 °C whereas the other dyes which do not have an azo bond dye the cloth at 100 °C. Also, they do not fade away easily and have four times more intensity than their alternatives (Khan and Malik 2013). But these dyes

are hard to degrade. Hence, they are harmful to the environment as they may colour the river making it aesthetically unpleasant and lead to its toxic contamination. They also affect the ecosystem by altering the pH and increasing COD and BOD, thus polluting environment. They form a coating on the surface of water bodies that does not allow the penetration of sunlight and exchange of gases causing the death of aquatic animals and plants. The dyes may also have substances which inhibit the photosynthesis process and hence can directly damage the autotrophs (Ventura-Camargo and Marin-Morales 2013). The dye disposal in water bodies will not only negatively impact the drinking water for humans and animals but also would affect the tourism, farming and fishing. Azo dyes affect the seed germination and inhibit the elongation of roots and shoots. It may cause metabolic stress, neurosensory damage and death in fishes. Moreover, if they didn't degrade, they may accumulate in the fishes (Chung 2016). The human body has reduction enzymes in digestive tracts and organs which reduces the azo dyes into aromatic amines (Benkhaya et al. 2017).

Below is the equation which shows the cleavage of azo dye in the presence of reduction condition, for example, in presence of sodium dithionite ($Na_2S_2O_4$) into aromatic amines:

$$\text{A-N} = \text{N-B} \xrightarrow[\text{pH 6}]{Na_2S_2O_4} \text{A-NH}_2 + \text{B-NH}_2$$

Twenty-four carcinogenic aromatic amines have been confirmed till now and hence are known to cause cancer, especially liver and bladder cancer. Five percent of azo dyes are recognised to be reduced to form such dangerous compounds. According to a 1992 study, exposure of aromatic amines such as benzidine, 2-naphthylamine and 4-amino biphenyl is known to elevate bladder cancer. Tobacco also has 2-naphthylamine which explains why the people consuming tobacco suffer from bladder cancer (Khan and Malik 2013). The azo dye which upon cleavage yields 1,4-diamino benzene (aromatic amine) can cause contact dermatitis, skin irritation, chemosis (swelling of conjunctiva of eyes), lacrimation (excessive flow of tears), exophthalmos (bulging of eye out of the socket), permanent blindness, hypertension, gastritis, vertigo, rhabdomyolysis (muscle injury which leads to kidney failure), acute tubular necrosis, vomiting and upon ingestion, leads to oedema of the tongue, neck, face, larynx and pharynx. Malachite green (Azo dye) is known as multiorgan toxin which damages the kidney, liver, heart and spleen. It is also known to hamper the food intake which leads to improper growth and also decrease the fertility rate (Sudha et al. 2014).

15.3 Industries Associated with Dyes

The growth of chemical industries is majorly contributed by the dye industries. Dyes are in use in various companies such as leather, paper, textile companies, carpet and garment companies, food, drug and cosmetics industries, etc. Dyes have large

applications from aluminium and plastic coatings to agricultural uses. There exist 100,000 marketable dyes which are accessible, and annual production of dyes is about one million tons, from which 10% are discharged in environment as wastewater effluents (Maguire 1992). Whereas, 50% initial dye load of reactive dyes is discovered in dye bath wastewater (An et al. 2002). Extensive use of dyes is mostly done by textile industries due to their minimal energy consumption, variety of colour shades and ease of application. The manufacturing processes of these industries are problematic caused by the release of harmful wastewater effluents containing large quantity of dyes. Textile industries discharge about 200 tons of dyes as effluent every year. This wastewater constitutes threat to environment, soil fertility and cause pollution and serious damage which can disturb the photosynthetic action of hydrophytes as well as are dangerous to aquatic organisms. Generally, 60–70% of the dyes in the world consist of azo dyes (Carliell-Marquet et al. 1995). Since, it contains N-N double bond, it can tolerate chemical attack, but researchers found that the process of three azo dyes by the human microflora gives potentially carcinogenic aromatic amines like aniline, 4-nitroaniline and 2,4-dimethylaniline (Khan and Malik 2013). It is imperative to separate the dyes from the contaminated water effluents because azo dyes and their break down outcomes are mutagenic, poisonous to human and toxic to aquatic life. The techniques to separate the dyes from wastewater is categorised in to chemical, physical and biological method. Biological processes are considered as the most efficient and environment-friendly as compared to other two methods (Karthik et al. 2014).

15.3.1 Conventional Way of Wastewater Treatment Containing Dye

There are many dyes removal techniques which can be employed to separate harmful dyes from the effluents discharged by textile industries such as Fenton reaction, nanofiltration, flotation, coagulation, electrochemical oxidation, photocatalytic degradation, membrane filtration, flocculation, sedimentation, equalisation, chemical oxidation, ozone oxidation, reverse osmosis, adsorption, membrane separation and homogenisation, aerobic treatment, anaerobic treatment, anoxic treatment, etc. (Fig. 15.6) (Karthik et al. 2014).

15.3.1.1 Physical Methods

Adsorption

Amongst all the removal techniques, adsorption process is popular because it is economical and readily available to control various pollutants from the wastewater. In this technique the porous material such as clay or activated carbon is mixed in the wastewater or the wastewater is allowed to pass through its filter bed which is made up of granular materials. The pollutants (dyes) get adsorbed on the porous filter and hence removed. Adsorbents such as polymers, ferric oxide and activated carbon are used. Adsorption is a selective process, i.e., different dyes show adsorption to selective adsorbents. In a series of adsorption reactions, the reduction of COD and

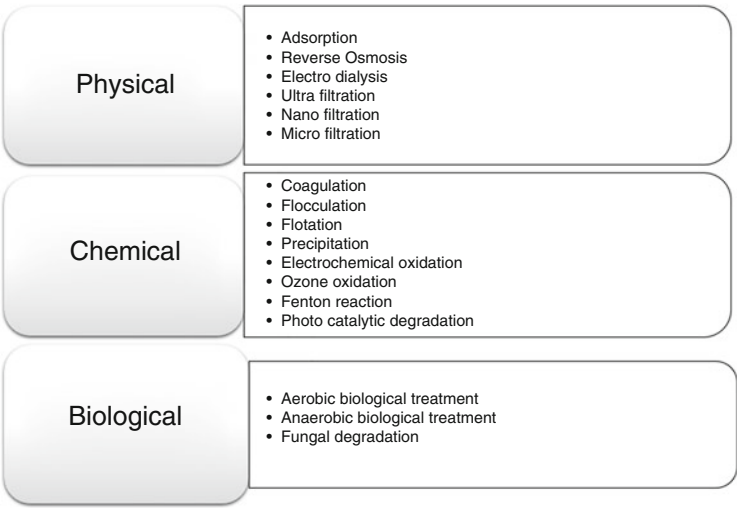

Fig. 15.6 Conventional way of wastewater treatment containing dye which includes three methods—physical, chemical and biological

removal of chroma to approximately 90% is reported (Sivamani and Beslin 2009). Dyes which are water dispersible, e.g., reactive dyes, azo dyes and basic dyes can be removed with ease by activated charcoal but it's inapplicable in removing insoluble dyes and suspended solids. Charcoal has a disadvantage, i.e., high cost of regeneration (Karthik et al. 2014).

Reverse Osmosis

In this process a partial permeable membrane is employed to separate ions or contaminants from the water. The solvent is coerced to move through a permeable membrane in converse direction owing to hydrostatic pressure applied which is superior to the osmotic pressure. Up to 90% of retention rate is accomplished by reverse osmosis membranes resulting in superior quality of permeate for many ionic compounds. Bleaching out and wash out of pollutants from wastewater can be executed in one step via reverse osmosis. Reverse osmosis can separate mineral salts, chemical auxiliaries and hydrolysed reactive dyes (Abid et al. 2012).

Ultrafiltration

In this process liquid is coerced to pass via semipermeable membrane bearing on hydrostatic pressure. The permeate achieves high purity and low slit density. Ultrafiltration helps in removal of viruses, colloids and other pathogens. The filter aperture is about 1 nm–0.05 μm so the dye removal process stays half-done therefore such treated water is not applied for delicate operations (Aouni et al. 2012)

Nanofiltration

This technique when used can treat coloured wastes released by textile industries. The pore size present in nanofiltration membranes is about 1–10 nm which is finer than the ultra and microfiltration membranes but is larger than that of reverse osmosis membrane. The membrane could retain small organic molecules, divalent ions, dyeing auxiliaries and reactive dyes (Abid et al. 2012).

Microfiltration

Membrane aperture is roughly 0.1–1 μm. This process is effectively used in treating of pigment dyes from dye baths. Microfiltration can be performed before nanofiltration or reverse osmosis for easier process (Taghdiri 2017).

15.3.1.2 Chemical Method

Electrocoagulation

It is the process in which iron (Fe) or aluminium (Al) as soluble anode undergoes electro dissolution resulting in formation of metallic hydroxide flocks in wastewater. In 2004, researchers performed electrocoagulation of reactive blue 19 dye in wastewater using Fe electrodes. Factors like initial dye concentration, electrolyte pH, applied voltage or current density, electrolysis time, temperature, supportive to electrolyte concentration were examined carefully (Karthik et al. 2014). The optimal settings for the dye removal was observed to be at pH 11.5, 50 ma/m^2 current density, 100 mg/l dye concentration, 20 min electrolysis time and room temperature, and supporting electrolyte concentration of 5 g/l NAD. 99.6% dye was removed under these conditions (Anantha Singh and Ramesh 2013).

Coagulation Flocculation Sedimentation

It is one of the most used techniques in traditional treatment process. The property of activeness on suspended matter prevents the aggregation due to the electric charge repulsion. To eliminate the surface electric charge on colloids, ferric chloride and aluminium sulphate are added in water which further gives organic hydrolysable polymers or hydrolysable metallic ions. This method is termed as coagulation. The coagulating agents are cationic in nature showing positive charge in water and generally colloids have negative charges (Verma et al. 2012). The particle gets aggregated due to the organic polymers and metallic hydroxides which increase the sedimentation. This process which involves the action of settling, coagulation and flocculation is called clariflocculation. Separate reaction tanks are required to perform this process because flow velocity and stillness is needed for settling. Heterogeneous matter undergoes mechanical separation but dissolved matter cannot be removed effectively in this method. Solubilised materials can be purified by other physicochemical or biological processes (Gadekar and Ahammed 2016).

Flotation

Substances formed may be in three phases, i.e., solid, gas and liquid, due to the large production of microbubbles (Mavros et al. 1994). Tiny bubbles are fixed to the particles while the air is being dissolved under pressure. Effects of bubble rising, buoyancy, hydrostatic pressure, interfacial tension and other forces help the microbubble and fibres to get attached present in wastewater. Mixture with low-density floats on the surface thereby dividing the oil drops from water. By this way the fibres can be easily removed from the wastewater (Dafnopatidou and Lazaridis 2008).

Electrochemical Oxidation

Researchers performed an experiment on removing the dye indigo carmine from wastewater using electrochemical oxidation via horizontally oriented electrode in a cell. The device contained Pb as anode and cathode as stainless steel. Complete decolourisation of dye and CO_2 reduction of 88.2% was achieved under optimum operating conditions (El Ashtoukhy 2013).

Ozone Oxidation

Ozonation can oxidise COD and inhibit the residual surfactant's foaming property. It converts the recalcitrant pollutants into easily biodegradable intermediates (Baban et al. 2003). Conventionally sodium hypochlorite was used as an oxidising agent because it cleaves azo bond in textile effluents, but it also led to release of carcinogenic aromatic amines.

Photo Catalytic Degradation

It is an advanced process of oxidation which mineralises the dye compounds. Titania catalyst is irradiated with light energy. This light energy causes the shifting of valence bond electrons to conduction band. Molecular oxygen scavenges the electrons present on the conduction band which are trapped by radicals of hydroxyl (Muhd Julkapli et al. 2014). These radicals have very high potential of oxidation; therefore, it is named as advanced oxidation process (AOP), as a result which oxidises the pollutants. Method of secondary disposal is not needed which is a good advantage as compared to other techniques. Recently researchers demonstrated the decomposition of methylene blue using calcium oxide by photo catalysis (Taghdiri 2017). Factors such as effect light intensity, pH, dye concentration and amount of calcium oxide were studied. As a result, removal of COD was achieved (Karthik et al. 2014).

15.3.1.3 Biological Method

Biological wastewater treatment removes dissolved pollutants very effectively than clariflocculation. The efficiency of this method is determined by the ration between biomass in oxidation tank and organic load, oxygen concentration and its temperature. The concentration of biomass is increased by aeration (Bhatia et al. 2017). It is important to examine the aeration as it should not reach the mixing energy which inhibits the settling and destroy the flocks. Generally, the concentration of biomass is

2500–4500 mg/l and 2 mg/ml for oxygen. Almost 99% reduction of oxygen demand can be achieved by 24 h aeration. Depending upon the oxygen demand, biological treatment is classified in to anaerobic and aerobic treatment. Aerobic biological treatment has wide applications and high efficiency; therefore, it becomes the major stream of biological treatment (Katheresan et al. 2018).

15.3.2 Mechanism of Dye Degradation with Aerobic Bacteria

A broad range of anaerobic and aerobic bacteria alike *Escherichia coli*, *Clostridium* sp., *Morganella* sp., *Alcaligenes* sp., *Xenophilus* sp., *Enterococcus* sp., *Klebsiella* sp., *Micrococcus*, *Dermacoccus*, *Aeromonas* sp., *Staphylococcus* sp., *Bacillus subtilis*, *Rhodobacter* sp., *Pseudomonas* sp., *Geobacillus* sp., *Rhizobium* sp., *Lactobacillus* sp., *Proteus* sp. and *Corynebacterium* sp. can degrade azo dyes as reported by number of scientific groups (Saratale et al. 2011). Azo dyes are consumed as source of nitrogen and carbon by some strains of aerobic bacteria (Gao et al. 2018).

Azo reductase is a major enzyme which catalyses the dye degradation under aerobic conditions. Azo dyes are reduced by some aerobic microorganisms which form intermediate metabolites like aromatic amines which can be degraded further to achieve azo dye mineralisation. The ability of aerobic microbes to destroy dye chromogens and reduce azo linkages is less than that of anaerobic bacteria; therefore, aerobic treatment has proven ineffective in many cases (Sarkar et al. 2017). Dye degradation is more effective when coupled anaerobic and aerobic treatment is employed (Feigel and Knackmuss 1993). Azo bond experiences cleavage in anaerobic conditions to form aromatic amines and in aerobic conditions through ring cleavage of azo bond by non-specific enzymes. Researchers demonstrated bacterial species which can effectively grow in aerobic culture, but dye removal was more efficiently obtained in anaerobic or anoxic culture. Stabilisation of dye metabolites have been successfully obtained in aerobic sludge. Few years ago, several bacterial strains which can break down azo dye have been isolated (Saratale et al. 2011).

Navitan Fast Blue S5R, a market textile dye was broken down by *P. aeruginosa* under aerobic conditions in existence of glucose. There are bacteria which can also grow on azo compounds solely, cleave –N=N– bonds and exploit amines as the carbon or energy source for their growth. Such organisms are substrate-specific, for example, *Pigmentiphaga kullae* K24 and *Xenophilus azovorans* KF 46 grows aerobically on carboxy-Orange II and carboxy-Orange I, respectively (Nachiyar and Rajkumar 2003). These microorganisms are unable to grow on structurally analogous sulfonated dyes, AO7 and Acid Orange 20.

15.3.2.1 Mechanism of Azo Dye Reduction

This involves various operations such as small-sized redox mediators and chemical reduction with the help of biogenic reductants such as sulphide and enzymes. The reactions may occur either extracellular or intracellular (Alabdraba and Bayati 2014).

- Direct Enzymatic Azo Dye Reduction
  Organic substrates oxidation gives rise to reducing equivalents. These equivalents which are reducing are shifted to the azo dye by enzymes. This is the first mechanism of azo dye reduction biologically. Azo dye reduction is yield by either specialised or common enzymes. Azo reductases are specialised enzymes which have an important role in reducing the azo dyes. Azoreductases were isolated from aerobic bacteria and found to be intracellular with good specificity to dye structures. Non-specific enzymes were isolated from aerobic cultures of *E. coli*, *Shigella dysenteriae* and *Bacillus* sp. and found to be flavoproteins (Suzuki 2019).
- Indirect Enzymatic Azo Dye Reduction
  This is the second metabolism of azo dye reduction in which enzymatically reduced electron carriers indirectly reduce azo dyes. Flavin-dependent reductase produces Flavins ($FNNH_2$, riboflavin, $FADH_2$) which can reduce azo dyes (Russ et al. 2000). Flavins stimulate azo dye reduction. Reduced enzymes cofactors NADH and NADPH are capable of reducing Azo dye directly. Many important artificial redox mediating compounds stimulate azo dye reduction (neutral red, phenosaphranin, benzyl viologen, FAD, 2-hydroxy-1,4-napthoquinone, methyl viologen, FMN, riboflavin) (Suzuki 2019).

Chemical Azo Dye Reduction

Dye-containing effluents undergo decolourisation due to the addition of reducing agents. Hence, chemical reductants like zerovalent iron and dithionite can be used to reduce azo dye. Sulphide, a biogenic reductant, can also reduce azo dye (Weber and Adams 1995). Sulphate concentration in dye is formed due to the oxidation of reduced sulphur species which are employed in dyeing processes like dithionite. Alkaline dye effluents are neutralised with sulphuric acid, thereby resulting in sulphate (Verma et al. 2012).

Aerobic Treatment on Aromatic Amines

Specific microorganisms are required for degradation of aromatic amines and type of biomass used also influences the process. After bioaugmentation with an appropriate bacterial culture, the degradation of sulfonic acid and aromatic amines can be achieved (O'Neill et al. 2000). In many experiments performed on degradation of aromatic amines, it is unpredictable whether the removal is caused by the adsorption, biodegradation or chemical reactions (Baird et al. 1977). Aromatic amines undergo auto-oxidation which results in slight decrease in colour. Researchers demonstrated the decolourisation of alizarin yellow R (AY) in a process combined with aerobic bio-contact oxidation and iron-carbon microelectrolysis (Liang et al. 2012). Under optimum conditions the degradation efficiency was greater than 96.5% and total organic carbon (TOC) removal rate was 79.44%.

15.4 Microbial Fuel Cells (MFC)

MFC is a device that converts organic matter directly into electrical energy using microorganisms as biocatalysts. In this system, the microbes feed on the organic azo dye and reduce them into aromatic amines (colourless) anaerobically and then degrade them aerobically since aromatic amines cannot degrade under anaerobic condition due to high redox potential (Jung and Pandit 2019).

15.4.1 MFC Components

Different materials are used to make the MFC components in Table 15.1.

15.4.2 Mechanism for MFC's Working

There are two types of MFC depending upon the number of chamber.

15.4.2.1 Single-Chamber MFC

Single-chambered MFC has an anode covered with microorganisms that oxidises substrate (like acetate) into CO_2 which leads to the production of electron and H^+ ions. The electron passes through the external circuit to reach the air cathode (Fig. 15.7). This produces electricity. The anode is separated from air cathode by a gas diffusion layer (GDL), and hence the H^+ ions produced reach cathode by diffusion and only passive oxygen is transferred to the cathode (Mathuriya 2014). But this eliminates the requirement for intensive energy aeration which is required in the dual chamber. This electron and H^+ ions are responsible for breaking the dye present in the chamber. This O_2 which is present at the cathode acts as an electron acceptor which combines with H^+ ions and electron to form water (Jung and Pandit 2019).

15.4.2.2 Dual Chamber MFC

In Fig. 15.8, there are two chambers shown—anodic and cathodic. The anodic chamber does not have oxygen and hence is anaerobic (Pandit and Das 2018). In this chamber the microbes are grown. The microbes utilize the substrate (like

Table 15.1 Different materials that are used to make the MFC components

Items	Materials
Cathode	Carbon paper, Pt black, graphite felt, Pt, reticulated vitreous carbon (RVC), graphite, carbon cloth
Anode	Graphite, carbon cloth, RVC, graphite felt, Pt, carbon paper, Pt black
Proton exchange system	Porcelain septum, salt bridge or solely electrolyte Polyethylene, sulphonated polystyrene, Nafion, Ultrex, poly (styrene-co-divinylbenzene)

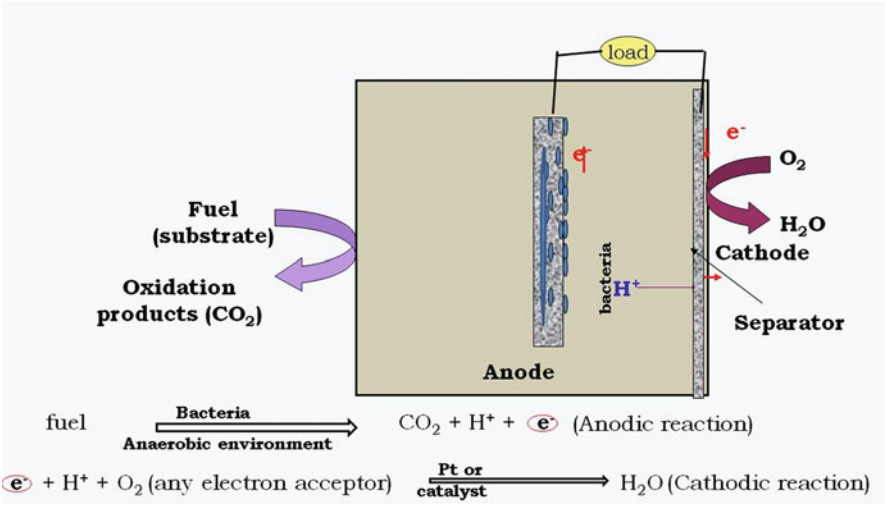

Fig. 15.7 Single-chambered MFC

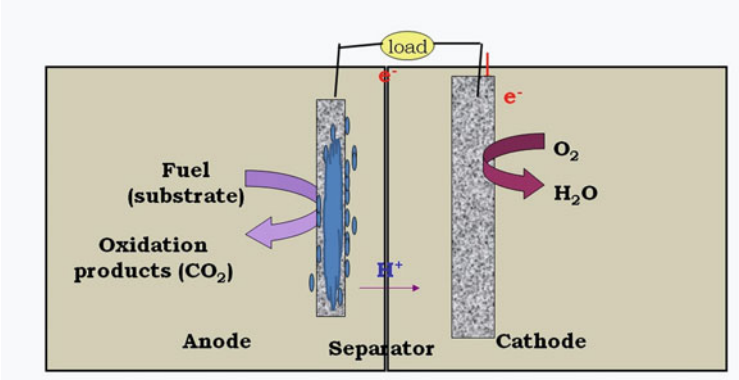

Fig. 15.8 Dual-chambered MFC

glucose) as their source of nutrient for their growth and forms CO_2, H^+ ions and electrons. The microbes along with the substrate also utilise azo dye as their nutrient source which leads to the decolouration of azo dye due to the breaking of azo bond into aromatic amines (Mathuriya 2014). The electron travels to the anode first which then moves to cathodic chamber via an external circuit (Fig. 15.6). There are two methods by which the produced electrons can be transported to the anode—indirect and direct.

In indirect method,

(a) Some external mediators like thionine or potassium ferricyanide must be added which can carry the electron to the anodic surface.

(b) The microbes excrete mediators which transfers the electron to the anodic surface.

In direct method, the microbe itself travels to the anodic surface and then through the redox enzymes present in its outer surface, transfers the electrons.

After reaching the anode, the electron flows through the external circuit to the cathodic chamber which leads to current generation. The cathodic chamber has oxygen and hence forms an aerobic environment (Pandit and Das 2018). H^+ ions from anodic chamber move to the cathodic chamber via IEM (ion exchange membrane).

15.4.3 Mode of Action of Dye Degradation Using Microorganisms in MFC

In mixed consortia, some microorganisms have enzymes in their outer membrane which actually breaks the azo dye into colourless aromatic amines. Examples of such enzymes are laccase, azoreductase, manganese peroxidase, lignin peroxidase and hydroxylases. Laccase enzyme is a copper oxidase enzyme that catalyses the cleavage of the aromatic ring of the azo dye by nucleophilic attack of water which results in the formation of phenolic compounds (Sudha et al. 2014). Some microbes use dye as energy/carbon source in anode chamber, during oxidation of these substrate, they donated electrons directly to anode and generate negative potential (Fig. 15.9). Another way of reduction of azo dye can be performed at the cathode chamber of MFCs.

15.4.4 Advantages of MFC

There are several advantages of MFCs over conventional treatment system, Some of them are: source of power generation, reduces pollution in environment, cuts the cost of water treatment, degraded dye instead of adsorption/piling up and hence safe disposal. Following are a few case studies on bioelectrochemical dye degradation in MFC (Pandit and Das 2018).

15.4.5 MFC Performance

The performance of MFC is highly affected by some physical and biological factors (Fig. 15.10)(Pandit et al. 2017).

15.4.5.1 Physical Parameters

For improvising the performance of MFC, selection of an appropriate separator and electrode material, which also accord in the expenditure of the MFC set-up is important.

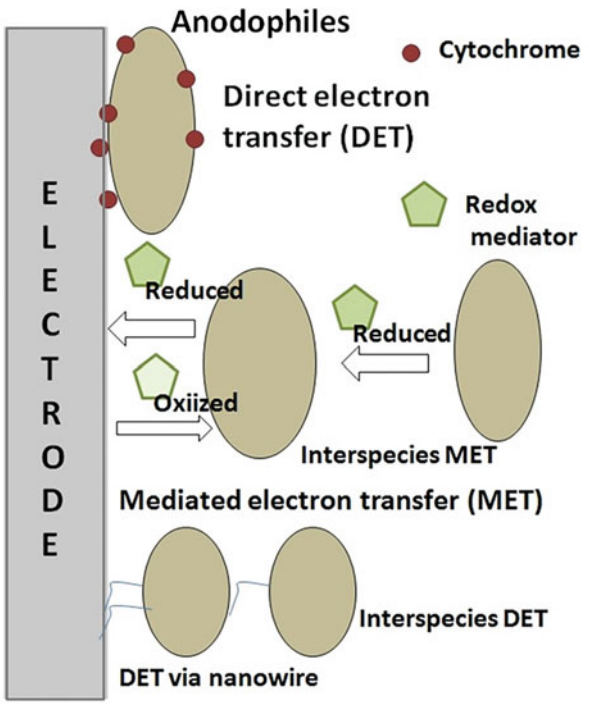

Fig. 15.9 Different mode of exocellular electron transfer by exoelectrognic bacteria

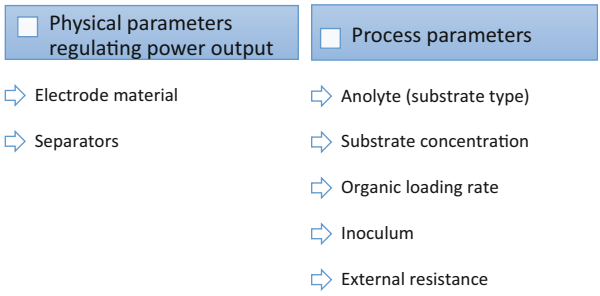

Fig. 15.10 Factors affecting MFC performances

Electrode Materials

Anode Materials

Attributes like chemical stability, high conductivity, attrition resistance ability, biocompatibility and better mechanical stability represent an ideal anode material. For better bacterial adhesion, anode must possess a huge surface area to volume ratio. Use of carbon-based anode substance is most preferred due to its easy availability, anticorrosive nature and low cost for the anchorage of electroactive bacteria (EAB). Graphite rod, reticulated vitreous carbon (RVC), felt and carbon cloth, etc. are examples of frequently used materials (Winfield et al. 2011). Carbon cloth reflects as a superior material due to its high porosity and flexibility that indeed

allows more attachment of bacteria, but its high cost limits the large-scale application (Pandit et al. 2014). Investigators have proved electricity production and improved sulphide removal acquired with activated carbon cloth due to its high S/V ratio and adsorption capacity. The 3-D electrodes are very successful in consequent power generation and better attachment of electroactive microbes (Scott and Yu 2015).

Non-carbon Anode Material

Dumas et al. applied stainless steel electrode for sediment MFCs where 4Mw/m^2 was maximum power density. A set-up of MFC-containing gold-based anode using a biocatalyst, *Geobacter sulfurreducens* resulted in generation of a steady current ranging from 0.4 to 0.7 mA (Zhou et al. 2011). But usage of gold in MFC at large scale is economically unfeasible (Wei et al. 2011).

Anode Surface Modifier

The major aim for modification of anode is to initiate changes in the chemical and physical properties in order to attain efficient electron transfer and improved microbial attachment. The electrical connections linking the electrode surface and bacteria and bacterial attachment are simulated by the surface characteristic of anode material. Modification methods involve: (1) use of metal-graphite compound electrode, (2) surface treatment via physical or chemical techniques and (3) addition of electroactive coatings (Zhou et al. 2011). Surface charge of anode is enhanced efficiently by ammonia treatment, thereby promoting current-generating biofilm. Immobilization of active amalgams on the carriers can be carried out for chemical modification of anode. Electron mediator like anthraquinone-1, 6-disulfonic acid can be used to obtain efficient improved convey of electrons from bacteria to anode. Acid treatment and heat treatment are some of the approaches for surface modification of anode (Peng et al. 2013). These methods result in removal of impurities and enhancement in conductivity and active site of electrodes.

Cathode Material

Cathode performance plays a markable role in producing power in MFCs. Graphite plate, carbon paper, felt and cloth are applied as cathode materials (Rismani-Yazdi et al. 2008). There is restriction on commercial application of non-catalysed materials due to their cathode reduction kinetics. The overall performance of MFCs declines due to the lethargy of reduction reaction (HaoYu et al. 2007).

Cathode Surface Area

Cathode's surface area is most significant to attain treatment efficiency for organic removal and better power output. After a certain threshold, increase in surface area is insignificant to generation of power output for a particular MFC system condition. Generally, more active site for oxidation reduction is provided by large surface. Forth one order of betterment in power output can be gained by increase in cathode surface by 11-fold using a double-chambered MFC (Zhou et al. 2011).

Cathodic Electron Acceptor (EA)

Cathodic activation over potential can be minimised with the use of alternate EAs. Various types of EAs are permanganate, dichromate, hydrogen peroxide, ferricyanide, bleaching powder, etc. EAs are oxidants with higher redox potential (Ucar et al. 2017). These EAs are unable to revive by oxidation with oxygen; therefore, application of such EAs is restricted (Wei et al. 2011).

Cathode Catalyst

Modification of cathode undergoes addition of Pt which is a highly active catalyst to raise the performance of MFCs. This increases the reaction rate and lowers down the cathodic reaction activation energy. A power density of 150 m/Wm^2 is obtained by the use of Pt as cathode containing graphite felt, and this density is thrice the density obtained by pure graphite electrode as studied by the researchers. Pt may cause sulphide poisoning during wastewater usage, and it is also very expensive; therefore, practical application of Pt is not economically viable (Sawant et al. 2016).

Operating Condition in the Cathode Chamber

Cathode half-cell potential is influenced by several operating parameters like buffering capacity of catholyte, catholyte pH, EA concentration in cathode chamber, temperature, etc. Reduction kinetics at cathode in two-chambered MFCs is improved by raising the level of oxidants as demonstrated by Yazdi et al. (Rismani-Yazdi et al. 2008). Oxidant concentration is proportional to the increase in reaction rate in the cathode chamber. Cathode performance increases by adding acid in aerated tap water, thereby decreasing catholyte pH. The reduction efficiency can be increased by facilitation of operating temperatures in the cathode chamber (Zhou et al. 2011).

Separators

A Separator in MFC is needed to minimise the gap between anode and cathode, avert short circuit via shielding of the electrodes and inhibit oxygen diffusion. The use of separators with high cation transfer property is necessary to increase the power output from MFCs. Examples of variety of separators are J-cloth, salt bridge, microfiltration membrane (MFM), cation exchange membrane (CEM), bipolar membrane (BPM), anion exchange membrane (AEM) and ultrafiltration membrane (UFM) (Li et al. 2011). The separators are classified in to 3 major categories depending on their characteristics: IEMs, salt bridges and size-selective separators. Although in absence of separator, the single-chambered MFC have increased current density, result in substrate diffusivity, increase in oxygen and consequent reduction of coulombic efficiency (CE) (Dhar and Lee 2013). Application of separators is partially advantageous as many problems need to be tackled so far. One major problem is about pH splitting in which there is a decrease in pH of anodic chamber and increase in the electrolyte pH of cathodic chamber. This declines the bioelectrochemical performance and system stability. This also increases the total cost of MFC operation and the overall internal resistance (Pandit et al. 2012).

15.4.5.2 Process Parameters

Substrate Type

The performance of MFC highly depends upon the nature of substrate. It is easier to degrade when the substrate is glucose, sucrose or acetate but harder when it's a complex organic matter such as real wastewater (Pant et al. 2010). Acetate is the best substrate as it is known to be inert to other biological processes such as fermentation (Li et al. 2009).

Substrate Concentration

The substrate present in the anode chamber gets oxidized and gives out electron. This electron is transported to the anode electrode which further travels via outermost circuit to produce current. Therefore, higher the concentration of substrate more would be the oxidation rate by the bacteria and larger will be the amount of current produced (Pant et al. 2010).

Organic Loading Rate

Organic loading rate (OLR) is a crucial factor for conversion of the substrate in the anodic chamber. Increase in OLR results in higher power generation but only when it is increased to an extent. If it is increased beyond the limit, it will inhibit the growth of microorganism which will result in the poor power generation (Abdelgadir et al. 2014).

Inoculum

The power generation performance of MFC depends upon the microorganism used as inoculum as this microbe is the reason for oxidation of the substrate and for the transfer the electron to anode for current generation. There are two types of inoculum used—pure culture and mixed culture (Jung and Pandit 2019).

Pure Culture

When compared with the mixed culture, pure culture can produce higher current. This is because the pure culture can produce a thin layer on anode which is conductive in nature, whereas the mixed culture inoculum is nonconductive and hence increase the resistance for charge transfer (Pandit et al. 2017). The power generation time required is less in pure culture and also only the selective microbes in mixed culture have the capability to transport electron to the anode. But having said that that the pure culture has its own challenges like easy microbial contamination and specificity for the substrate. For large-scale degradation and generation of electricity, mixed culture is used as they are easily available, and they are more tolerant to the environmental changes. The most commonly used pure culture for MFC is of *S. putrefaciens* or *G. sulfurreducens* (Roy and Pandit 2019).

Mixed Culture

The mixed culture requires more time to start the electricity generation and also more time is needed to acquire a stable power output. After a certain period of time, i.e.,

after acclimatisation, mixed culture's active consortia are selected by MFC for power generation. These microbes usually belong to γ-proteobacteria or α-proteobacteria (Fan and Xue 2016).

External Resistance

External resistance is a vital factor for generating power in a MFC. The higher the external resistance, the higher is the voltage and lower is the current produced. Because as stated by Ohm's law.

$$R = \frac{V}{I}$$

Where, R = Resistance, V = Voltage, I = Current.

The more the energy gained by the microorganism, the more will be the oxidation of substrate, the more will be the electron transportation on anode and hence the more will be the electricity production. At low external resistance, positive potential is generated on anode which results in the microorganism to take more energy and hence more power generation (Pandit et al. 2015).

15.4.6 Evaluation of Performance of MFC Components

15.4.6.1 Evaluation of Anode Performance

Anode performance can be modified using different materials for anode construction (Manohar et al. 2008). This can be evaluated by using different electrochemical methods like- chronoamperometry (CA), electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) and chronopotentiometry (CP) (Martin et al. 2013).

15.4.6.2 Evaluation of Cathode Performance

The cathodic chamber receives electron from the external circuit. The chamber already has oxygen present. This oxygen acts as electron acceptor and thus gets reduced. It is a crucial step in MFC which requires a catalyst to improve its efficiency (Kircheva et al. 2015). The reduction catalyst, hence, is required to be evaluated. Evaluation can be done using linear sweep voltammetry (LSV), EIS and CV (He and Mansfeld 2009).

15.4.7 MFC Reactor Configuration

The components of MFC can be modified for achieving better output. These modifications are made for creating a suitable design for MFC reactor. This reactor layout greatly influences the coulombic efficiency and power output (Premier et al. 2015). Different types of configurations are as follows:

15.4.7.1 Double-Chambered MFCs

Dual-chambered MFC is suitable for wastewater treatment. However, the input energy in association with driving fluid is huge than the output power. The quantitative relation between anolyte volume and high electrode surface area and less spacing in the middle of electrodes promotes high power density. It is difficult to scale up dual-chambered MFCs because of their large volume, high internal resistance and complex design (Premier et al. 2015).

15.4.7.2 Single-Chambered MFCs

Single-chambered MFCs have many advantages; hence, it is more preferred over double-chambered MFCs. The advantages are as follows: (1) Easy handling due to the smaller volume. (2) High volumetric power density due to arrangement of short electrode spacing. (3) Oxygenating cathode chamber consumes zero power as usage of submissive air is done. (4) Operations as chemical revival of catholyte or recycle are nonessential (Premier et al. 2015). Air cathode membrane-less MFCs have simple configuration, high power density with compact reactor and low cost (Nancharaiah et al. 2016). Nevertheless, coulombic efficiency is low as compared to single-chambered MFC with membrane.

15.4.7.3 MFCs with Multielectrode System

Supplementary pathway for transfer of electrons and increment in the tempo of reduction reaction is provided by MFCs with multielectrode system. About three times power density is produced by single-chamber MFC containing four pairs of cathode and anode which forms a multielectrode system as compared to the MFC with single pair (Sonawane et al. 2013).

15.4.7.4 Stacked MFCs

A series connection of various fuel cells adds the voltage, while all fuel cells contain the common current flow. A parallel connection of fuel cells increases the current output and maintains the voltage (Shin et al. 2006). The combination of appropriate number of parallel and series connection of power sources or fuel cells can help in achieving any desired current or voltage (Zhuang et al. 2012). The series connection produces six times lower current as compared to the parallel, which proves that series connection of MFCs will restrict high current density as reported by Aelterman et al. (2006). Parallel connection of MFCs is appropriate for high current density and rapid substrate degradation as reported by their study (Aelterman et al. 2006).

15.5 Case Studies of Simultaneous Azo Dye Removal and Electricity Generation

In reality, various physicochemical methods like flocculation and coagulation are practiced; nevertheless, these techniques failed to make an impact owing to extra prerequisite for disposal technology along with large volume sludge production.

Expenditure associated with cost and large energy utilisation limits the processes like ozonisation and advanced oxidation process and UV/peroxide application. The slow kinetics of dye degradation rate with current biological treatment method like anaerobic reduction process makes it non-sustainable. Furthermore, requirement of co-substrates increases the cost of operation, and it is responsible for greenhouse gas generation. The enzymatic treatment for dye degradation was found difficult due to product inhibition and chemical instability of enzyme. MFC was found effective in removing dyes from contaminated water along with bioenergy harvesting (Solanki et al. 2013).

15.5.1 Cationic Dyes

Cationic dyes can be defined as the dyes which can dissociate into positively charged ions in the presence of an aqueous solution. They are also amongst the earliest synthetic dyes discovered. They are also called as basic dyes and possess a positively charged chromophore, i.e., the coloured region (Benkhaya et al. 2017). These dyes interact with the negative groups on the fibre and bind to it forming a salt and dyeing the fibre as a result. They are mostly used in polyester and acrylic fibres. Cationic dyes are of different types such as azo dyes, heterocyclic compounds triarylmethane dyes, and anthraquinone dyes. Cationic dyes can be degraded by a few biological fungi species such as methylene blue degraded by Aspergillus sp., multi-walled carbon nanotubes (MWCNTs) are used for rhodamine B dye which gives up to 90% dye adsorption. Another method used for cationic dye degradation is photodegradation or photooxidation (Topare 2013). The majority of dyes employed in the textile industry are anionic dyes since most fabrics are cationic and anionic dyes can better bind to them with electrostatic interactions.

Han et al. in 2013 used MFCs for the degradation of methylene blue and simultaneous generation of electricity. Wool, cotton and silk fibres are common materials used for dyeing with methylene blue. Its presence in wastewater can lead to severe conditions in humans such as nausea, diarrhoea, etc. They also used gold nanoparticles as catalysts of the microbial activity and checked the outcome in the presence and absence of gold nanoparticles. They found the maximum electricity production to be 36.56 mW/m^2 and methylene blue was completely degraded simultaneously. With respect to the use of gold nanoparticles, they found that 98% of methylene blue and 96% of the COD had been separated in the presence of gold nanoparticles, whereas only 57.4% of methylene blue and 40% of COD had been separated in the non-existence of gold nanoparticles (Han et al. 2013).

Jumma Shaikh in 2016 had successfully demonstrated the degradation of the cationic azo dye, methyl red. They had used a two-chambered MFC with *Bacillus circulans*. They had achieved the maximum electricity generation of 710 mV at 200 ohms and the maximum decolourization was found to be 98%. A maximum power density of 856 mW/m^2 was produced after the incubation of 48 h (Shaikh et al. 2016).

Cheng et al. (2014) were able to successfully degrade and simultaneously generate electricity from crystal violet. They worked with a single-chambered MFC for the degradation, inoculated with an exoelectrogen *Aeromonas hydrophila* YC 57. The removal efficiency achieved was 82.5 ± 0.7% and coulombic efficiency was 57.2 ± 0.5% at initial crystal violet concentration of 100 mg/L and the maximum power generation of MFC was 240 ± 5.6 mW/m^2. Further, they found the optimal pH range to be 6–8, which showed an average of 96.5% degradation efficiency and the optimal temperature span to be 25–35 °C, the optimal temperature span to be 25–35 °C, which showed an average of 92.5% degradation efficiency for crystal violet degradation by *Aeromonas hydrophila* YC 57. They also noticed that the coulombic efficiency and removal efficiency both lessen with the increase in crystal violet concentration (Cheng et al. 2014).

15.5.2 Anionic Dyes

Li and Jia (2008) proposed dual-chambered MFC in which cathode was made up of carbon felt and graphite granule, whereas the anode was composed of carbon felt. The anaerobic chamber was introduced with the wastewater of Congo red dye which then broke down to aromatic amines. This was then transferred to the cathode chamber to degrade it. Different concentrations were used. 14.8 h was required for the electricity generation and was found to be the optimised time. The percentage for the colour removal of the azo dye was found to be 69.3–92.7% at a concentration of glucose—4000 mg/l and at glucose concentration 1000 mg/l, electricity generated was 387 mW/m^2 (Li and Jia 2008).

Sun et al. used single-chambered MFC in which the anode was made up of non-wet proof carbon paper, whereas the cathode was made up of wet proof carbon paper which had 0.5 mg/cm^2 Pt coating. The chamber was filled with Congo red, and power generated was found maximum at 900 mg/L of concentration (Sun et al. 2011b).

Hou et al. (2011) worked with MFC in which the anode was composed of non-wet proof porous carbon paper, whereas the cathode was composed of wet proof carbon paper which had 0.5 mg/cm^2 Pt coating. Different types of membranes were used like proton exchange membrane, microfiltration membrane and ultrafiltration membrane with the molecular weight cut-off 1, 5 and 10 K. The chamber was filled with Congo red dye for colour removal and power generation. High density power was generated—324 mW/m^2 and the ultrafiltration membrane of 10 K showed the highest efficiency rate for colour removal (Hou et al. 2011).

Ding et al. (2010) worked with dual-chambered MFC in which anode was made up of unpolished graphite, whereas the cathode was made up of polished graphite. Anaerobic sludge was launched into anodic chamber and the cathodic chamber with electrolyte which was the replaced with methyl orange azo dye. Only 37.8% of colour was removed when graphite electrode was used. 47.7% of colour was removed when rutile electrode was used but when rutile electrode was irradiated

by visible light, the colour removal was found to be 73.4% with maximum production of current (Ding et al. 2010).

Liu et al. (2011) used a two-chambered MFC to reduce methyl orange dye with electrons produced in situ in the MFC, i.e., without any external supply of electricity. They prepared the bio-anode with activated fibre of carbon and introduced electroactive bacteria which formed biofilm on the anodic chamber (Liu et al. 2011). The cathode was built of carbon paper or modified with thionine and anthraquinone-2, 6-disulfonate (AQDS). They monitored the degradation process with the help of UV-electroscopy, 465 nm was found to the maximum absorbance of methyl orange. The dye strength reduced with the reaction time. They reached a maximal power density of 1.3 mW/m^2 with a very low coulombic efficiency.

Fu et al. (2010) proposed dual-chambered MFC in which the anode was made up of granular graphite, whereas the cathode was made up of spectrographic pure graphite. Amaranth azo dye was introduced into MFC. The author gave two different Fenton system—electrochemical and conventional. The power generation in the electrochemical was found to be higher, i.e., 28.3 W/m^3, whereas in conventional, it was 11.1 W/m^3, but the dye removal in conventional Fenton system was higher, i.e., 82.59%, whereas in the electrochemical, it was 76.43% (Fu et al. 2010).

Zhang and Zhu (2011) worked with single-chambered MFC in which 25 g graphite rod was the anode, whereas the cathode was constructed of carbon paper which had 0.5 mg/cm^2 Pt coating. Acid Orange 7 azo dye was introduced into the MFC. The maximum power generated was 5 W/m^3, and 97% of colour was removed in 168 h (Zhang and Zhu 2011).

Thung et al. (2015) suggested one-chambered MFC in which the anode was composed of carbon felt whereas the cathode of carbon plate. The MFC was introduced with a mixed culture of anaerobic sludge for the remedy of Acid Orange 7 azo dye. The maximum voltage output was found to be 148.4 ± 17.3 mV. The total COD removal and colour removal was 90% (Thung et al. 2015).

Sun et al. (2009) used one-chambered MFC in which the anode was made up of porous carbon paper, whereas the cathode had 0.5 mg/cm^2 Pt layer on that part of the cathode which was facing the water and the other part which was air facing had polytetrafluoroethylene (PTFE) diffusion layer. Three MFCs were prepared. To each chamber, active brilliant red X-3B was introduced with different types of carbon source in each—glucose, sucrose and acetate. It was established that the greatest power density was produced in the MFC which had glucose and the lowest was produced in acetate fed MFC. 100% of colour was removed using dye concentrations—300 and 600 mg/l and the time required was 48 h (Sun et al. 2009).

Sun et al., worked with dual-chambered MFC in which both the electrodes were composed of porous carbon papers which were separated using a PEM. Active brilliant red X-3B azo dye was introduced into the MFC. The colour removed was 81.56%, and the power generated was 50.74 mW/m^2 (Sun et al. 2011a).

Fang et al. (2013) worked with CW- MFC in which *Geobacter sulfurreducens* and *Betaproteobacteria* were introduced with active brilliant red X-3B azo dye of 150 mg/L concentration. The colour removal rate was known to be 91.24%, and the voltage produced was 610 mV (Fang et al. 2013).

15.6 Challenges in MFC Operation for Dye Degradation

As a lot of research is going on for dye degradation using MFCs, few challenges have also come up such as low coulomb efficiency, the high cost of proton exchange membrane. And if replacement by cheaper cation/anion membrane is done, it results in low-power yield. Another issue is being able to find the perfect electrode design in manufacturing MFCs to make it a cost-effective technology and cheaper alternatives to noble metals, which are used as catalysts. In recent years, various types of carbon and materials made of metals have been investigated for the development of anodes and cathodes, and various modification techniques for the electrodes have also been elaborated. Some studies also found that increasing the biomass led to better discolouration but resulted in a lower electricity generation (Kim et al. 2007). Also, cases of membrane fouling and low rate of growth of microbes limit the MFCs' applications. Amongst the different types of reactor designs, the two-chambered MFCs have shown the highest internal resistance and hence high-power consumption. Showed high internal resistance and increased energy consumption. Therefore, single-chambered MFCs are better suited for azo dye degradation and electricity generation because of its advantages (Radhakrishnan and Jayaprakash 2017).

The electrode price and the amount of power generation have still not reached the level for commercial usage, even after the electrode layout being progressed from planar to a concrete structure. This is the same reason why MFCs have not been able to be used on a larger scale. As an alternative to noble metals used as a catalyst, biocathodes have been designed in which certain microorganisms act as catalysts. One such example is the use of granular activated carbon (GAC) biocathode as an alternative to noble metals like platinum. GAC provides increased surface area for biofilms which are very important for MFC operation and also cost-effective (Zhang et al. 2012). Therefore, MFCs have lots of advantages like the simultaneous generation of power and dye degradation, reducing CO_2 emission and sludge generation, etc.; the challenges such as expensive materials, current instability, high internal resistance and low electricity production used pose as barriers to its complete utilization.

15.7 Conclusion

This chapter overviews the industries associated with dyes and the wastewater treatment mechanism which involves decolourisation of harmful dyes. It focuses on various mechanisms for the breakdown of dyes with the assistance of aerobic and anaerobic bacteria. This chapter also discusses different case studies for cationic, anionic and non-ionic dyes with MFCs, factors affecting dye degradation and challenges in MFC operation. Furthermore, it also discusses the materials used in MFC, the different types of dyes, their structure and effects along with the advantages and disadvantages of the dye degradation methods. From, this chapter, it can be concluded that MFC can perform biodegradation of azo dyes with very

good results. However, the power density needs to be improved in MFC and also there is a requirement for low cost-efficient component materials which will be suitable for the scale-up study of the wastewater treatment. In future, there is a good scope for biocathode technique and nano-coated electrodes which will facilitate performance result as regards to power density and decolourization of dye.

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Psychrophiles as the Source for Potential Industrial Psychrozymes

16

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Abstract

Temperature is one of the foremost imperative environmental factors for life because it impacts most biochemical response. It has directly accompanied by the changes in gene expression, membrane fluidity, protein conformation and stability reaction kinetics. During the past two decades, studies on low-temperature organisms have been accelerating the interest in research of multicellular vertebrates, invertebrates, bacteria and algae from deep sea, ocean, glaciers and Polar regions. These psychrozymes have shown high catalytic activity at low and moderate temperatures. The research on cold-active enzymes mostly concentrates on pharmaceuticals uses, food processes technology, bioremediation and antifreeze proteins. This present study focused on the briefing of the residual modification and structural changes for the adaptation of the psychrozymes. In details, we discussed the molecular strategy for cryo-defense by psychrophilic bacteria and their potential industrial applications. Thus it will be assumed that in the near future the psychrophiles would be a potent source for cold-active enzymes and might be used for the cost-effective production of the industrial important biocatalyst.

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16.1 Introduction

Temperature is a major determining factor that affects all living organisms and has played a significant characteristic for the selection and distribution of microorganisms on our planet. In the recent past, the microorganisms that thrive at harsh environment gained more research interest because of their application in biotechnological processes and their importance in understanding the molecular basis of their adaptation (Whitman et al. 1998). Mercury in 1974 first proposed the name "extremophile" referred to the organisms that survive and proliferate under which may be considered as the fatal environmental conditions compared to the physico-chemical characterization of the normal environment. Based on the environmental conditions, the organisms are grouped and distinguish into seven families of extremophiles, such as (a) psychrotrophs (proliferate at 0–30 °C) and psychrophiles (at 0–20 °C and easily thrive at below 0 °C); (b) thermophilies (readily grow at 60 °C); (c) halophiles (completely dependent on salt concentration for their proliferation); (d) piezophiles (successfully colonized in environment having the high hydrostatic pressures); (e) metallophiles (adapted to survive in high concentration of heavy metals); (f) acidophiles (easily can survive at pH value of 2) and (g) alkaliphiles (survive under alkaline condition at exceeding pH values of 9–10).

During the past two decades, studies on low-temperature organisms have been accelerating the interest on the research of multicellular vertebrates, invertebrates, bacteria and algae from deep sea, ocean, glaciers, and Polar regions (Morita 1975; Feller 2003). However, psychrotrophs survived at both moderate and freezing temperature thus considered it as ubiquitous. The temperature shifts are directly accompanied by the changes in gene expression, membrane fluidity, protein conformation and stability reaction kinetics. The psychrozymes are portrayed as competent for high-synergist movement at low and moderate temperatures; be that as it may, those cold dynamic catalysts are heat-labile and quickly inactivated at a mellow temperature. Psychrophilic enzymes can be up to multiple times increasing the active at low and moderate temperatures as contrasted and their mesophilic homologues. Thus much attention is given to the identification and isolation of cold-loving microorganisms and its biocatalysts (Chattopadhyay 2006). These particular characteristics are liable for the three primary preferences of cold-active enzymes in biotechnology. At a lower concentration of the catalyst, it is required to arrive at a given action, consequently diminishing the measure of costly enzyme preparation in a process, and, as a result of heat-lability, they can be effective, and, in some cases, specifically inactivated after a handle by direct warm input (Gerday et al. 2000).

16.2 Briefing of the Initial Exploration of Psychrophiles

In 1840, the botanist Hooke conducted the first naval discovery explorations of Antarctica and suggested the evidence of cold-adapted microorganisms; indeed, 4 years later, Certes demonstrated the growth of bacteria in low temperatures. Foster who had isolated bacteria from a cold-adapted fish, measured their growth at low temperature. In 1887 and later in 1892, Forster had isolated bacteria from various habitats and especially from seawater which was capable of proliferating at 0 °C. In 1888, Fischer had grown certain pathogenic bacteria at 0 °C; however, the name of "psychrophiles" was first coined by Schmidt-Nielsen in 1902.

In the same year, Feitel isolated denitrifying bacteria from deep seawater and proliferated them at low temperature (Duman et al. 1991). In that continuity, Conn in 1910, Brown-Smith in 1912, Vanderleck in 1918 and Vass in 1919 isolated microorganisms and incubated them at low temperature. The psychrophilic bacteria from the marine environment were reported by Eimjhellen and his group in 1964. In 1959 Ingraham and Bailey suggested that the psychrophiles grow rapidly at 0 °C. However, the redefined of psychrophiles, those bacteria not only grew rapidly at or below 0, 15 and 20 °C (Feller and Gerday 2003). Some microorganisms are most frequently colonized in cold environment such as all permanently cold environments from the deep sea to the mountain and Polar regions denoted as psychrotolerant microorganisms (Wynn-Williams 1990; Aislabie et al. 2004).

16.3 Strategy for Cryo-Defense by Psychrophilic Bacteria

Temperature is one of the foremost imperative environmental factors for life because it impacts most biochemical response. Investigation has been in advance to uncover the biochemical and molecular instruments of different microorganisms that can offer assistance to set up the warm sensitivities, ideals and resilience limits of, in an unexpected way, adjusted species and, in an unexpected way, acclimated or acclimatized populaces of a single species (Reeves 1977).

The adaptive response of psychrotrophic bacteria is capable of developing over a wide temperature range and even at temperatures close to or below freezing. In extreme temperatures, two main components are needed to adapt: the membrane and the enzymes. The lipid cell membranes and the cellular matrix of cold adaptive organisms are chemically resistant due to the 'antifreezes' protein, even protect in temperatures below water's freezing point (Chattopadhyay 2006). These low temperatures and freezing conditions may impact the lives in numerous ways by changes in membrane smoothness, diminished biochemical response rates, expanded thickness of the medium, the ability to reproduce successfully, nutrient availability, confirmative change in the cellular protein and need for protection against freezing (Reeves 1985). Subsequently, to preserve an ideal smoothness in low temperature, a number of changes are happening within the fatty acid profile of bacterial cell membrane (Chattopadhyay 2006).

It is well established that microorganisms increase the level of unsaturated fatty acid in the membrane phospholipids response to the shifting of temperature from

high to low. The fluidity of the cells is adjusted by shortening the length of fatty acids and by the introduction of unsaturated and branched fatty acid chains. This phenomenon is known as *homeoviscous adaptation*. The microorganisms have the sense that helps them to adjust to the environmental temperature shifting (Arpigny et al. 1997). To understand the response of upshift and downshift of temperature, conducted the in vitro and in vivo experiments on Antarctic membrane protein bacterium *Pseudomonas syringae* for phosphorylation and dephosphorylation. He observed that lipopolysaccharide (LPS) which is found in the outer leaflet of the membrane bilayer undergoes structural changes in the core region by phosphorylation and dephosphorylation with the shifting in temperature. From the primary investigation, it was found that in *P. syringae* the composition of the acyl chain in LPS differ from low to high temperature (Chattopadhyay 2006).

16.3.1 Cold Acclimation Proteins and Antifreeze Proteins

Investigations on cold adoring life forms uncovered the insider facts of cold adaptation at genomic as well as proteomic level. The cold shock domains in psychrophiles genomes are reported for putative roles in RNA stabilization. The study on *lhkA* mRNA of *L. monocytogenes* demonstrated the up expression during the growth at a cold temperature (Liu et al. 2008). The degradosome, a complex of proteins found in the psychrophilic organisms, helps the stability of cellular RNA and thus cold adaptation of psychrophilic bacteria. However, it was also suggested that eliminating the necessity of ATP might help the cell in the conservation of energy at low temperature (Purusharth et al. 2005).

Cold shock spaces (CsdA) that relate with 50S precursors are recognized as a fundamental multifunctional protein at low temperatures, which is included in the biogenesis of the 50S ribosomal subunits (Radjasa 2004). It has been observed that during transcription CspA proteins binds to nascent mRNA which prevents the formation of intramolecular hydrogen bonds of RNA chain and thus facilitates coupling of transcription to translation. Psychrophiles exhibited many diverged kinds of proteins including cold shock protein (CSP) or class I protein and cold acclimation proteins (CAP) or class II proteins. The classification of class I and class II proteins such as CspA, CspB, CspG and CspI, ribosome-binding protein (RbfA), transcription factor NusA, RNA helicase CsdA and exoribonuclease PNPase, histone-like protein H-NS, translation initiation factor IF2α, trigger factor, recombination protein RecA, DNA gyrase subunit A, pyruvate dehydrogenase subunit E1 and dihydrolipoamide dehydrogenase, respectively (Villeret et al. 1997). Homologues of CspA proteins are widely distributed in all psychrotrophic bacteria including those from Antarctica. Amongst them, the CspA family of ~7.0 kDa small acidic proteins constitutes the most common type of proteins found in bacterial species (Violot et al. 2005).

When the temperature drops well below 0 °C, psychrophilic organisms require specific adaptive strategies in order to maintain membrane fluidity, the continuance of their metabolic activities, and protein synthesis at low temperature that can protect

the organisms to survive against the fatal condition, such as synthesis the antifreeze proteins (AFP), which act as to depress the freezing point or prevent the recrystallization or develop cryoprotectants or formations of ice-nucleating proteins, and synthesized cold-shock proteins or Csps (de Vries and Cheng 1992; D'Amico et al. 2002). The antifreeze protein molecules found in the Antarctic fish is considered as a glycoprotein of three amino acids Ala-Ala-Thr. It is bound to a disaccharide (Suutari and Laakso 1994). Later five other AFP was also discovered from Antarctic fish species. Surprisingly, although these proteins don't have any common structural characterization, all of them play a common role to depress the freezing points. In addition, cold-adapted microorganisms, fungi, insects, plants and even some vertebrates have developed additional cryoprotectants such as glucose, glycerol, sorbitol, amino acids, trehalose and various derivatives that enable them either to avoid freezing or even to tolerate freezing. These molecules mainly act by depressing the freezing point through collective effect, rapid redistribution across the membrane. It can help to protect proteins from cold denaturation by preventing the dehydration of cells. The organisms could produce a small protein named as ice-nucleating proteins (INP) when the condition drops in foetal temperature (Yamashita et al. 2002). INP act as paradoxical for the organisms by forming ice on an extracellular layer at a temperature above the normal freezing point. The idea is to preserve the intracellular space from freezing and allow the organism to cope with ice by controlling its temperature of formation (Pulicherla et al. 2011).

16.3.1.1 Concept of Cold-Active Biocatalyst at Low Temperatures

According to Morita, the term 'psychrophilic enzymes' should only be used for enzymes isolated from psychrophilic organisms. Later, Brenchly coined the word 'cold-active' for the enzymes that have exhibited the highest catalytic activity at low temperature. However, it is well considered that both 'cold-active' and 'cold-adapted' are better terms than 'psychrophilic'. Ohgiya classified and characterized cold-active biocatalysts as heat-sensitive enzymes that are similar to mesophilic enzymes; the second category is known as heat-sensitive as and relatively more active than mesophilic enzymes at low temperature; and the third group is thermostable as mesophilic enzymes but more active than mesophilic enzymes at low temperature (Georlette et al. 2003; Mavromatis et al. 2002).

Cold-adapted from the bacteria at cold environment results in the two possibilities such as, growth at low temperature which involved in synthesizing new proteins and/or cellular proteins having hot and cold stable properties to function normally at low temperature (Mazur 1977). Apart from that, to maintain the sustainable activity at low temperature, the cryophiles follow two other strategies for existing in low temperature; these involve synthesizing cold-adapted enzymes that enhanced the catalytic efficiency (k_{cat}/k_m). It is well-known that in most enzymes, both k_{cat} and k_m increase with respect to temperature (Gilbert et al. 2005). By increasing $k_{cat,}$ and decreasing k_m or by changes in both parameters can influence the catalytic efficiency (Smalas et al. 2000).

In the cold-adapted enzyme, the substrate-binding region has found to be the most flexible region when the unfolding starts due to its high k_m. In contrast to mesophilic

enzymes, the enzymes from cold-adapted bacteria have high-conformational flexibility including the reduction of electrostatic noncovalent weak interactions decrease of hydrophobicity, which would be responsible for their increased catalytic efficiency and their low thermal stability. Now according to the activated complex theory, the *Arrhenius equation,* the rate of a chemical reaction is directly proportional to the absolute temperature and influenced by the magnitude of $\Delta G^{\#}$ for the reaction. However, in constant temperature, the reaction rate will be indirectly proportional to ΔGs. The free energy of activation ($\Delta G^{\#}$) is independent of enthalpy of activation ($\Delta H^{\#}$) and entropy activation ($\Delta S^{\#}$). In the case of psychrozymes, the activation enthalpy of reaction is lower than the mesophilic and thermophilic enzymes which can be considered as the main adaptive character and may cause to increase flexibility and lower entropy activation.

The stability of cold-adapted enzymes relate to the basic components is capable of the stability of the three-dimensional structure of the protein by structural alternation (Russell 2002; Lelivelt and Kawula 1995). The numbers of arginine and proline residues are reduced, and the clusters of glycine residues provide localized chain mobility in cold-adapted enzymes (Feller and Gerday 2003).

16.3.2 Structural Adaptation of the Psychrozymes

The interest towards the cold adaptive organisms is exhibited from the rapid growth in a number of sequencing and characterization of cold adaptive enzymes. To date, crystallographic structures psychrozymes include six from bacteria and four from fish have been elucidated. However, out of ten enzymes, unfortunately, a structural difference of citrate synthase, α- amylase, malate dehydrogenase, xylanase, triosephosphate isomerase and trypsin have been compared with mesophiles and thermophiles homologous. The systematic investigations on structural parameters of psychrophilic enzymes have shown three main factors that are the causes of reducing the rigidity of the psychrozymes (Aghajari et al. 1998a).

First, compared with the mesophilic counterpart, the number of prolines is drastically low and the distribution of prolines in psychrophile was reported in loop regions compared with the mesophiles. The heat liability of psychrozymes is directly related to the prolines. Second, the reduction in arginine build-ups diminishes the number of inside electrostatic intelligent keeping up the by and large overlap and competent to make different salt bridges and hydrogen bonds, as well as a lower number of particle sets, and a debilitating of charge – dipole intuitive in a-helices (D'Amico et al. 2001). Third, the clustering of hydrophobic side chains inside the centre protein could be a major driving constraint of protein unfurling (Aghajari et al. 1998a; Charollais et al. 2004; Feller et al. 1994). The salt bridges in protein present in an approximate ratio of 4 per 100 residues (Hazel and Williams 1990). According to the sequence alignments, the variations have been observed in loop length that can impose uncertainties and will be the main cause to affect the protein structure.

16.3.3 Residual Sequences of Cold-Adapted Enzymes

It has been found that all amino acid residues of the active site of psychrophilic enzymes were involved in the reaction mechanism (Kim et al. 1999). The description of systematic comparative analysis of psychrozymes illustrated that the charged residues Glu, Arg and Lys tend to replace with Ala, Ser and Asn, respectively, occur mainly in the α-helices region. The change in charge residue would be a mechanism to adapt to the low temperature. In mesophile and thermophile, Arg to Lys substitutions are mostly present at high residuals in increasing numbers, and in psychrophiles the more frequently found residual is Asn (Lonhienne et al. 2000; Aghajari et al. 2002). For cryophilicity in protein, the main determining factor is the low ratio of Arg/Arg + Lys.

Furthermore, Val is highly preferred in β-stands of thermophiles and mesophiles, while in psychrophiles Val has found to be less frequent in the buried region and in β-stands (Aghajari et al. 1998b). Compared with the mesophilic, eight of the protein classes possess the low aliphatic residue such as Ile^+ Leu and Ile^+ Leu^+ Val ratio for the psychrozymes representative. Interestingly in cold adaptive trypsins, substitutions are found from Ile to Val, from Thr/Val to Ala and from Try to Phe, result in the disruption of internal hydrogen bonds. The ratio of proline is observed in less number in the loop region of the representative classes of psychrozymes, assumed that they are responsible for flexibility to achieve optimum enzyme (Chattopadhyay 2002; Qian et al. 1994). (Feller et al. 1998). The X-ray structure of cold-active α-amylase unravelled at a high determination as compared to its closest mesophilic auxiliary homologue. Most of the contrasts between psychrophilic α-amylases and mesophilic α-amylases are watched within the number and character of the circles (Clark et al. 2004).

16.3.4 Cold-Adapted Enzyme from Marine Psychrophilic Microorganisms

Around 71% of the earth's surface, i.e., 361 million square kilometres is secured by the seas which are accepted to contain add up of around 3.67×10^{30} microorganisms. According to ecological zonation of the deep sea, the psychrosphere occupied at least 90% marine environment, and their average annual temperature is in between 10 and 5 °C. This marine environment represents an enormous pool of potential microbial biodiversity that ranges from Gram-negative bacteria (e.g. *Pseudoalteromonas*, *Moritella*, *Psychrobacter*, *Moraxella*, *Psychroflexus*, *Polaribacter*, *Vibrio*, *Polaromonas*, and *Pseudomonas*), Gram-positive bacteria (e.g. *Micrococcus*, *Arthrobacter*, *Bacillus*), Archaea (e.g. *Methanococcoides*, *Methanogenium*, *Halorubrum*), Yeast (*Candida* and *Cryptococcus*) and Fungi (*Penicillium* and *Cladosporium*), collectively all these organisms revolutionized the cold marine biotechnology (Pulicherla et al. 2011). The research on cold-active enzymes mostly concentrates on pharmaceuticals uses,

food processes technology, bioremediation and antifreeze proteins (Margesin et al. 2005).

16.4 Application of Cold-Active Enzymes from Marine Psychrophilic

Utilize of chemicals in different applications is well-known for numerous decades and enzymatic strategies involve a critical and basic part in advanced mechanical forms. Nearly all bacterial proteins for the industrial items are confined from mesophiles and at the same time due to the heat-stability; the thermophilic chemicals are too habitually utilized in businesses as perfect biocatalysts (Margesin and Schinner 1994; Tomiyama et al. 2002). In a few industrial applications, the enzymatic responses need to be carried out at low temperature (Coker et al. 2003). In such cases, cold-adapted proteins can be more pertinent over mesophilic or thermophilic proteins. The benefits draw in consideration of businesses to treat their items with these cold adjusted enzymes (Pulicherla et al. 2011).

- Energy sparing: Cold-dynamic proteins can spare vitality by dodging warming and cooling steps during a process.
- Easy inactivation of chemicals amid post handling steps thermo-flexible proteins can be effortlessly inactivated in direct temperatures.
- Prevention of defilement: Food processing at low temperatures anticipates the development of mesophilic contaminants.
- Saving of labile or unstable compounds: In biotransformations of food processing, the unstable or labile compounds can be saved.

The applications of cold-adapted biocatalysts are summarized in Table 16.1. The chemicals such as α-amylase, protease, cellulase, and lipase are utilized for bio-cleaning and stone washing of material items, and it is additionally utilized in cleanser detailing businesses (Trevino et al. 2007). Squander water treatment by evacuating poisonous compounds such as nitrates, hydrocarbons, fragrant compounds, overwhelming metals and biopolymers, such as cellulase, chitin, lignin, proteins and triacylglycerols, can too be done by the cold adjusted chemicals.

Advantage of cold washing in cleanser industry diminishes vitality utilization and a diminishment in wear and tear within the cold washing. β-Galactosidase is broadly utilized within the concept of narrow lactose mindedness. Low action of β-galactosidase causes digestive lacking called lactose bigotry. Cold-adapted β-galactosidases might be utilized to decrease the sums of lactose in the drain as well as suppression of lactose crystallization in the sweet condensed drain and ice creams, expanded sweetness, and diminish within the hydroscopicity of dried dairy items.

Pectinase within the natural product juice industry has progressed the juice extraction prepare and decrease the thickness in items. Pectinase debases pectic substances and has endless utilization in businesses where the end of pectin is basic

Table 16.1 Sources and applicants of cold-active enzymes

Biocatalyst	Microorganisms	Applications
α-Amylase	*P. haloplanktis*	Washing reagent for clothes, paper coating manufacture of high fructose-containing syrups
β-Glucosidase	*Paenibacillus* sp.	Modification of flavours and enhancement I food material
β-Galactosidase	*Pseudoalteromonas* sp. TAE 79b, 22b *P. haloplanktis* TAE79 *Arthrobacter* sp. *Pseudoalteromonas* sp. *C. piscicola*	Dairy industries
Cellulase	*P. haloplanktis* TAC125	Formulation of detergent,
Catalase	*Pseudoalteromonas* sp. DY3 *P. haloplanktis* *V. salmonicida* *V. rumoiensis* S-1	Cheese production
DNA ligase	*P. haloplanktis*	Biological research
DNA polymerase	*C. symbiosum*	Biological research
Endonuclease I	*V. salmonicida*	Biological research
Esterase	*Psychrobacter* sp. ANT300, *Pseudomonas* sp. *Acinetobacter* sp. *Acinetobacter* sp.	Wastewater treatment, formulation of detergent
Lipase	*Pseudomonas* sp.	Formulation of detergent, wastewater treatment
Oxidase	*Pseudoalteromonas* sp.	Applied as the preservative at the food packing industry
Protease	*P. fluorescens* *P. issachenkonii* *C. psychrerythraea* *E. freundii* *Pseudomonas* sp.	Leather industries, formulation of detergent
Pectate lyase	*P. haloplanktis*	Fruit juice, paper industry
RNA polymerase	*S. violacea*, *P. syringae*	Biological research
Superoxide dismutase	*P. haloplanktis*	Pharmaceutical applications, clinical purposes

(Hoondal et al. 2002). The application of cold-adapted pectinase would permit to diminish the thickness of natural product juice at cold temperatures and strides the squeezing capacity of the mash causing the deterioration of jam structure; in this way the natural product juice is effortlessly gotten with higher yields (Margesin et al. 2005). This would spare the quality of the natural product juice and empowers inactivation of the protein at direct temperatures after treatment.

16.5 Conclusion and Future Aspect

In today's world, there's an expanding drift towards the utilization of renewable, cheap and promptly available microbial biomass within the generation of a wide variety of fine and bulk chemicals in numerous industries. Over the years, enzymes from thermophiles and mesophiles are dominating. Indeed, with the growing environment as well the industrial concerns, the interest in the sources of biocatalytic conversions have recently emerged. The research interest is sifted from terrestrial to the extreme environment. Catalysis at low temperature for an example is advantageous in the prevention of contamination and saving of labile or volatile compounds during food processing. In addition, the catalytic activity at low temperature can help to avoid the heating and cooling steps during a process, thus reduction of the production cost. To further illustrate in the uses of psychrophilic biocatalyst suggested the significant applications in large-scale industrial approaches. Despite many efforts, still today the often limited due to the discovery of the noble sources.

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Transcriptional Regulators *in Bacillus anthracis*: A Potent Biothreat Agent

17

Shikha Joon, Sonika Bhatnagar, and Rakesh Bhatnagar

Abstract

Transcriptional regulators are highly dynamic modulator proteins that bind to the specific DNA targets. These achieve their regulatory effects via activators or promoters. Consequently, the gene transcription is either upregulated or downregulated. These are known to be an integral component of the cell signaling and signal transduction cascade. CodY is a DNA-binding protein that regulates the transcription of several genes involved in crucial cellular activities. *Bacillus anthracis* is a potent biowarfare agent. Within the bacterium, the CodY targets include genes involved in metabolism, amino acid biosynthesis and transport, nitrogen assimilation, motility, biofilm formation, sporulation, and virulence. Owing to the vitality of the CodY protein in its anthrax pathogenesis, it becomes pertinent to broaden our horizon on its structural and functional attributes.

Keywords

Bacillus anthracis · Anthrax · Transcriptional regulators · Sporulation · Virulence

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17.1 Introduction

CodY is a prokaryotic transcriptional regulator present in Gram-positive bacteria with low G + C content in their genome. Its pleiotropism is well-demonstrated in the myriad cellular activities including, motility, competence, biofilm, and pellicle formation, and virulence in pathogenic bacteria (Ratnayake-Lecamwasam et al. 2001; Tu Quoc et al. 2007; Lemos et al. 2008; Majerczyk et al. 2010; Gopalani et al. 2016). CodY activity is governed by the intracellular concentration of GTP and Branched-chain amino acids (BCAAs). These effector molecules are known to stimulate the DNA-binding activity of CodY. Consequently, the genes under CodY regulation (e.g., sporulation and adaptation genes) are not transcribed. This usually occurs during the exponential phase of bacterial growth when the effectors are in abundance. On the contrary, the concentration of the effectors drops as the cell transits from the active to the stationary phase. This, in turn, abrogates the DNA-binding activity of CodY, and the genes under its regulation are rescued (Fig. 17.1) (Stenz et al. 2011).

The binding site and the mechanism of interaction for the BCAAs and CodY are well-documented in various homologs (Villapakkam et al. 2009). However, little is known about the GTP and CodY (*B. anthracis*, in particular) interaction mechanism. Further, the residues involved and the biochemical processes that occur post-binding remain elusive. Reportedly, CodY possesses putative GTP-binding motifs (G1, G2, G3, and G4) similar to those observed in the small GTP-binding proteins (Ratnayake-Lecamwasam et al. 2001; Handke et al. 2008). Although these residues

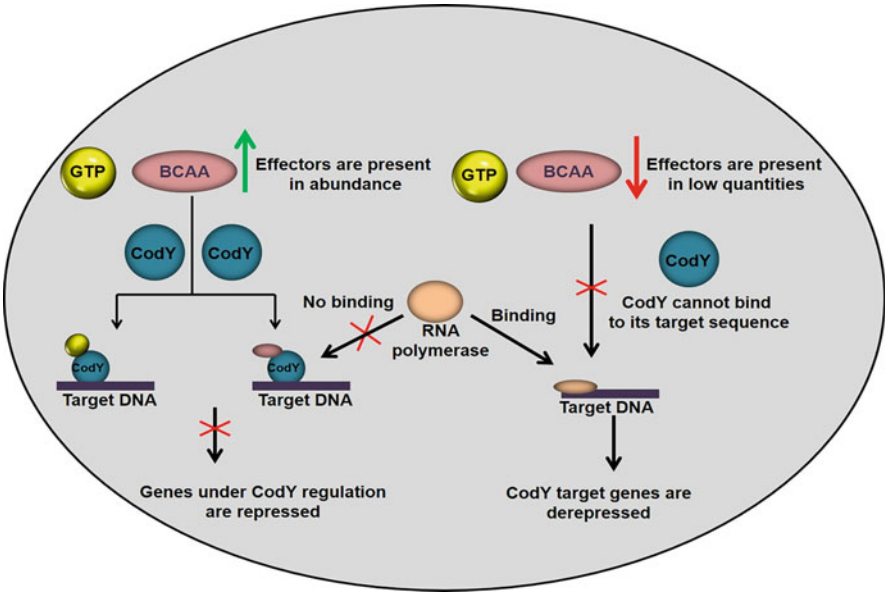

Fig. 17.1 The mechanism of CodY action and regulation

are conserved in CodY homologs, they do not participate in the GTP-binding. The same was ascertained by the crystal studies of CodY (of *Staphylococcus aureus*) bound to GTP. Accordingly, the GTP-interacting residues were observed in the Metabolite-Binding Domain (MBD) and the Long Helical Linker (LHL). These motifs were not observed in the vicinity of the binding site for GTP (Han et al. 2016). There are exceptions to this. For example, CodY of *Lactococcus lactis* and *Streptococcus pneumonia* does not interact with GTP at all (Petranovic et al. 2004; Hendriksen et al. 2008). It is, thus, desirable to assess this attribute in individually in all the CodY homologs and identify the crucial residues, in case the binding is observed. This is particularly important in the pathogenic bacteria as well as those posing a biothreat.

In *B. anthracis*, CodY regulates around 500 genes. This regulatory function is executed either directly or indirectly (Château et al. 2013). It has a well-documented role in the survival of this pathogen (Gopalani et al. 2016). Reportedly, CodY executes an indirect control over the virulence determinants in *B. anthracis.* Accordingly, AtxA protein (anthrax toxin activator) regulates the expression of virulence genes directly (Dai and Koehler 1997). It was observed that disruption of *codY* gene affected the virulence gene expression in vitro. There was a complete abrogation of virulence property of the bacterium. But, the *atxA* gene expression remained unaffected. Thereafter, the overexpression of the *atxA* gene restored virulence. It is, therefore anticipated that CodY might regulate the posttranslational accumulation of AtxA protein (van Schaik et al. 2009). In *B. anthracis*, the biochemical aspects of CodY and GTP-binding are unexplored, and the residues involved in this interaction are not deciphered yet.

17.2 A Brief Description of *Bacillus anthracis* and Anthrax

Bacillus anthracis is a Gram-positive sporulating bacteria that cause anthrax (Mock and Fouet 2001; Hudson et al. 2008). It is a zoonotic disease that occasionally affects humans. The bacterial spores can sustain in a hostile environment (soil) for decades (Nicholson et al. 2000). This form of survival continues until favorable growth conditions are met (Fig. 17.2). The tripartite toxin complex [protective antigen (PA), lethal factor (LF), and edema factor (EF)] and an anti-phagocytic capsule (poly-γ-D-glutamic acid) are the main arsenals of anthrax pathogenesis (Leppla et al. 1999). The spores enter the host system via cutaneous, gastrointestinal, or inhalational routes. This marks the initiation of the anthrax infection cycle (Goossens and Tournier 2015). As a host-immune response, the local macrophage population gathers at the site of infection. Unfortunately, they are hijacked and exploited by the bacterium for germination. The vegetative bacilli are formed which release their respective toxin components in the host system. Foremost, PA (83 kDa) binds to its receptor on the host macrophages. This is followed by cleavage of its 20 kDa fragment. 63 kDa PA molecule then oligomerizes and provides a scaffold for LF/EF binding. This binary complex (PA + LF/PA + EF) is now active. Further, it is internalized by receptor-mediated endocytosis (Gordon et al. 1988). Due to a low

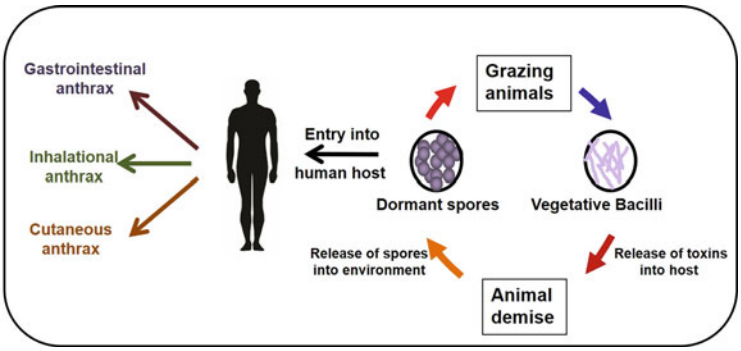

Fig. 17.2 Infection cycle of *Bacillus anthracis* and its pathogenesis

endosomal pH, the complex dissociates and lethal or edema toxins are formed (Blaustein et al. 1989; Milne and Collier 1993; Klimpel et al. 1992; Milne et al. 1994). Consequently, these enter the cell cytosol and upon accumulation, inebriate the host system, prompting demise (Barua et al. 2009; Lowe and Glomski 2012).

17.3 Bacterial Transcriptional Regulators

Transcriptional regulators are highly dynamic DNA-binding proteins. These display a stringent regulatory mechanism and functional pleiotropism (van Hijum et al. 2009; Hottes et al. 2013; Chesmore et al. 2016). Accordingly, they recognize and bind to the specific sequence of their target genes. These genes are known to code for the proteins involved in crucial activities such as intercellular signaling, cell cycle control, pathogenesis, etc. In general, the structural elements comprise a DNA-binding domain (DBD), the ligand-binding domain (LBD), and the trans-activating domain (TAD). As the name suggests, the DBD is responsible for promoter recognition and binding. Also, it harbors distinct DNA-binding motifs (Wintjens and Rooman 1996; Dahl et al. 1997; Wolfe et al. 2000; Laity et al. 2001; Vinson et al. 2002; Wärnmark et al. 2003; Piskacek et al. 2007). While the LBD senses the ligand/effector molecules, other coregulators are bound on the TAD. Consequently, the target genes are either expressed or repressed (Latchman 1997; Wärnmark et al. 2003; Piskacek et al. 2007). These "mobile switches" are, in turn, regulated by the posttranslational modifications presenting a stringent control mechanism (Fig. 17.3) (Beck-Sickinger and Mörl 2006).

Within the bacterial cell, these "modulators" work concertedly allowing for cellular adaptation in response to environmental cues (Galperin 2006; Sanchez and Demain 2008). *B. anthracis* is characterized by the presence of two important transcriptional regulators, anthrax toxin activator (AtxA), and capsule gene activator (AcpA) (Hoffmaster and Koehler 1999; Koehler 2002, 2009). While AtxA controls the expression of the virulence genes, AcpA is associated with the synthesis of the capsular genes in the bacteria. Other transcriptional regulators include NprR, SinR,

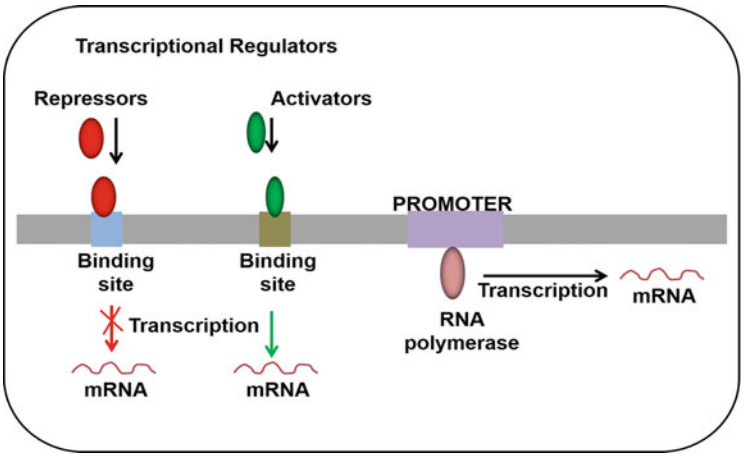

Fig. 17.3 The general mechanism of regulation by transcriptional regulators in bacteria

SigH, abrB, Bla/Mec, IclR, GntR, LysR, ArsR, and TetR. These regulate crucial mechanisms such as quorum-sensing, biofilm formation, virulence, sporulation, antibiotic resistance, etc. (Château et al. 2013). Owing to the fact that the transcriptional regulators are an integral part of the signal transduction cascade, these might serve as potential drug targets.

17.4 The Pleiotropic Regulator CodY in *B. anthracis*

17.4.1 Metabolism

Within the host, several factors influence gene transcription in bacteria. This is more prominent during infection, wherein host-specific environmental factors trigger a modification in the gene transcription of bacteria. For instance, during iron deprivation, the genes responsible for iron homeostasis are overexpressed (Chateau et al. 2011; Kim et al. 2016). Further, the CodY-dependent effects were evident in the expression of the genes involved in amino acid and siderophore biosynthesis. For example, in a *codY* deletion mutant, the ability to utilize heme iron was drastically reduced. This severely affected virulence in the pathogen (Cendrowski et al. 2004). Similarly, the central metabolism and the carbon overflow genes were under CodY regulation (Kim et al. 2016).

17.4.2 Sporulation

CodY is a part of the sporulation phosphorelay network. It works in concert with other transition state regulators. These include AbrB, SigH, etc.

(Ratnayake-Lecamwasam et al. 2001; Inaoka et al. 2003; Molle et al. 2003). During a period of active bacterial growth, CodY represses the sporulation genes. At this stage, the nutrients are available in abundance (Mitani et al. 1977; Lopez et al. 1979; Brossier and Mock 2001; Mock and Fouet 2001). Any reduction in the nutrient supply signals the concerned regulatory proteins. For example, CodY allows the expression of the sporulation genes (Spo0A, Spo0B, etc.) in case of nutrient scarcity. The genes for BCAAs synthesis are under direct CodY regulation. It was speculated that these might be critical for CodY activity (Slack et al. 1993). These observations suggest that nutrient deprivation and sporulation are interrelated (Richardson et al. 2015).

17.4.3 Virulence

Several protein factors govern the virulence trait in *B. anthracis.* Together, these form an intricate network and are regulated by the AtxA (a transcriptional activator) protein (Dai and Koehler 1997). This master regulator activates the toxin gene expression, namely, *cya* (lethal factor, LF), *pag* (protective antigen, PA), and *lef* (lethal factor, LF). The genes involved in the capsule synthesis, that is, *acpA* and *acpB*, are also under its regulation. These comprise the capBACDE operon (Dai et al. 1995; Dai and Koehler 1997). In a *codY* mutant, the toxin gene expression was drastically reduced. The posttranslational accumulation of AtxA protein was also affected (van Schaik et al. 2009). Also, *codY* disruption severely affected the virulence attribute of a toxinogenic strain in a mouse model of infection without affecting its capsulation (Château et al. 2013). These studies support the notion that CodY might be indirectly involved in the virulence in *B. anthracis*, though the mechanism of regulation unknown. Thus, CodY is a transcriptional regulatory protein with a cardinal role in vital cellular processes such as metabolism, biosynthesis, adaptation, and virulence in this pathogen (Kim et al. 2016).

17.5 Structure of CodY of *B. anthracis* and its Interaction with GTP

CodY of *B. anthracis* harbors highly conserved domains and motifs that allow its interaction with GTP. Specifically, there is a GAF-like domain (N-terminal) and the Helix-Turn-Helix domain (C-terminal). These are characteristic features of the transcriptional regulators (Brennan and Matthews 1989; Martinez et al. 2002; Joseph et al. 2005). Additionally, putative GTP-binding motifs (G1, G3, and G4) are observed in the sequence of CodY of *B. anthracis*. These are known to interact with GTP in the small GTP-binding proteins. Although these motifs are highly conserved among the CodY homologs, including *B. anthracis*, no functional significance could be ascertained (Bourne et al. 1991; Han et al. 2016). Further, the homology modeling revealed the presence of highly conserved residues in CodY of *B. anthracis*. These were also observed in its homolog in *Staphylococcus aureus*.

Together, these residues form a GTP-binding site comprising a "G" (E153) and a "P"- pocket (S43, R45, K47, Q70, and K158), respectively (Joon et al. 2017; Han et al. 2016).

Similar to a lot of eukaryotic and prokaryotic GTP-binding proteins, CodY of *B. anthracis* interacts with GTP and autophosphorylate itself in vitro. This occurs on a conserved Serine residue in the 215th position (Boguski and McCormick 1993; Takai et al. 2001; Malumbres and Barbacid 2003; Pandit and Srinivasan 2003; Joon et al. 2017). Mg^{+2} ions are utilized as a factor and are essential for this activity. The negative charge on the phosphate group oxygen (Nucleotide tri-phosphates) complexes with the Mg^{+2} and imparts a shielding effect. This facilitates a phosphoryl group transfer during an autophosphorylation reaction (Pilotelle-Bunner et al. 2009). A similar role is speculated in the case of CodY of *B. anthracis*. A much deeper insight into the autophosphorylation mechanism of this protein is required though. The sequence analysis revealed that $Serine^{215}$ lies in the winged HTH motif of CodY of *B. anthracis*. This residue is conserved throughout its homologs (Joseph et al. 2005). It is hypothesized that $Serine^{215}$ might play an important role in DNA-binding (Stenz et al. 2011). These findings on the involvement of $Serine^{215}$ in the autophosphorylation activity of CodY of *B. anthracis* together with its proven role in DNA-binding in its homolog are suggestive of an additional regulatory mechanism. To confirm the vitality of the $Serine^{215}$ residue in DNA-binding, further validation is required.

CodY of *B. anthracis* not only binds to the GTP but also cleaves it; GTP hydrolysis. Mg^{+2} is indispensable for the GTPase activity. Divalent cations are an essential component of the hydrolysis reaction undertaken by GTPases (Zhang et al. 2000; Pilotelle-Bunner et al. 2009). It has been reported that GTP hydrolysis leads to conformational changes in the protein. This, in turn, serves as a regulatory mechanism. Subsequently, a myriad of cellular processes such as ribosome assembly, protein synthesis and transport, signaling, and pathogenesis are regulated (Haller et al. 1997). Interestingly, the absence of GTP hydrolyzing activity in CodY of *B. subtilis* and *S. aureus* did not affect its affinity for the target genes in vitro (Handke et al. 2008; Han et al. 2016). Since scarce is known about these species-specific attributes of CodY of *B. anthracis,* it is not possible to conclude its functional relevance. Therefore, a detailed mechanistic understanding of this transcriptional regulator is needed (Fig. 17.4).

17.6 Conclusions

CodY is a multifaceted transcriptional regulator that steers a spectrum of cellular activities in various Gram-positive bacteria. The activity of CodY is governed by the intracellular levels of its effector molecules, namely, GTP and BCAA. Recent works on the CodY-GTP interaction led to the discovery of some species-specific attributes of this protein. Reportedly, CodY of *B. anthracis* exhibited GTP hydrolysis and autophosphorylation as unique biochemical attributes in comparison to its homologs. The presence of the conserved $Serine^{215}$ residue in the DNA-binding

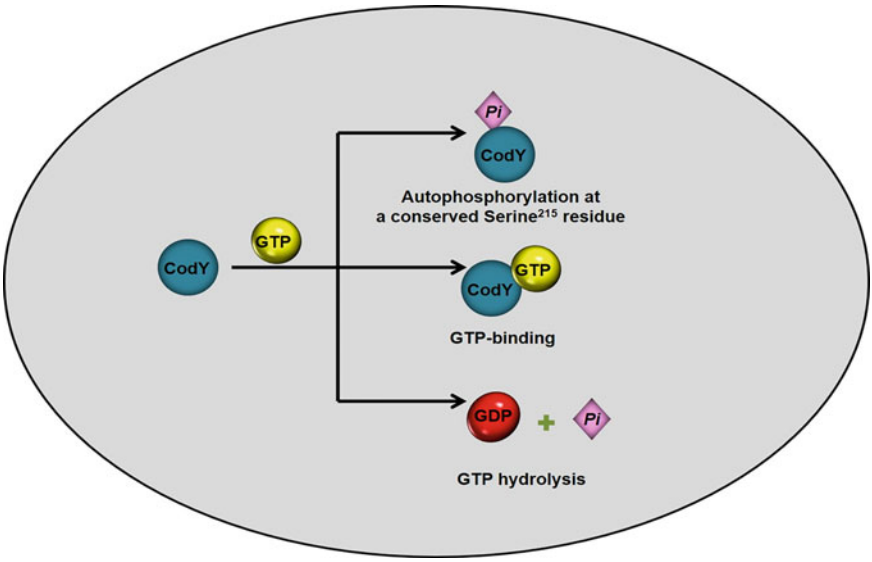

Fig. 17.4 A schematic representation of biochemical attributes of CodY of *B. anthracis*

domain might present an additional regulatory mechanism of CodY activity. Homology modeling of *B. anthracis* CodY revealed some crucial facts pertaining to the GTP-binding residues. Accordingly, the previously speculated GTP-binding motifs (G1, G3, and G4) were found to be spatially located distant from those inferred from *S. aureus* CodY. The conserved residues observed in the crystal structure of the CodY-GTP complex might be involved in the GTP-binding activity of *B. anthracis* as well. But, this must be validated by rigorous experimentation in vitro. Indeed, these findings have contributed to the understanding of the structure-function relationships in CodY, its mechanism of action of CodY, and regulation in *B. anthracis*.

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Medicinal Fungi: A Natural Source of Pharmacologically Important Metabolites

18

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Abstract

Medicinal fungi have diverse biological properties such as anti-inflammatory, anticancerous, antidiabetic, and antioxidative activities. Mushrooms are known to possess bioactive molecules, i.e., polysaccharides like β-glucans, triterpenoids, and antioxidants. These molecules are known to have therapeutic activities including immunomodulation. Among these medicinal mushrooms, species of *Ganoderma* like *G. lucidum*, commonly called as Reishi (traditional Chinese medicine), has shown a potential anticancer activity. Polysaccharides extracted from this mushroom show anticancer activity through immunomodulation. Chaga, *Inonotus obliquus*, is another mushroom been used as a folk medicine against cancer. *Cordyceps* is one of the most important health foods of humans, which grows on larvae of moths and converts each larva into a sclerotium, from which the stroma and fruit body grows. Another medicinal mushroom, *Phellinus linteus* containing Beta D-Glucan and lectin was shown to have immunomodulating effects. *Xylaria* is commonly known as dead man finger fungus, some of its species producing sesquiterpenes have been used as medicine for treating insomnia and depression. The purpose of this review is to summarize information regarding pharmacologically important compounds from medicinal fungi.

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Keywords

Ganoderma · *Phellinus* · *Cordyceps* · *Xylaria* · Anticancerous · Antidiabetic · Polysaccharides

18.1 Introduction

Mushrooms have been used as food and as a medicine all over the world from ancient times (Wasser 2011). Generally, mushrooms were categorized into three categories based on their uses, viz., edible, medicinal, and poisonous mushrooms. The market of countries like China and other western countries use a number of dried fruiting bodies as a herbal medicine. Moreover, pharmaceutical molecules isolated from medicinal mushrooms having potent health-boosting capabilities were distributed worldwide (Wasser and Weis 1999). Higher Basidiomycetes mushrooms are known to contain a large amount of well-balanced essential amino acids. These mushrooms also contains wide ranges of substances namely polysaccharides, compounds derived from the shikimic acid, aromatic phenols, fatty acid derivatives, polyacetylenes, polyketides, nucleosides, sesquiterpenes, marasmanes, hirsutanes, caryophyllanes, etc., diterpenes (cyathin, striatal), sesterterpenes (aleurodscal), and many other metabolites from different origin (Wasser and Weis 1999, Lorenzen and Anke 1998). Tissues of all mushrooms contain large amount of important fibers which are used for consumption. One of the bioactive molecules isolated from mushroom fruit-bodies, submerged cultured mycelial biomass, or liquid culture broths is polysaccharides which is either water-soluble β-D-glucans, containing heterosaccharide chains of xylose, mannose, galactose, or uronic acid, or β-D-glucan-protein complexes, i.e., proteoglycans (Mizuno 1999). Increased scientific and medical researches on these mushrooms further confirm the medicinal importance of compounds isolated from them and their bioactive efficacy (Wasser and Weis 1999; Ooi and Liu 2000; Hobbs 2000). The bioactive polysaccharides isolated from mushrooms are effective against various diseases and have properties like anticancer, immunomodulatory, hepatoprotective, antidiabetic, antiarthritis, antitumor, antifatigue, antistress, and hypocholesteromic. Many studies have been done on the medicinal mushrooms confirming the bioactive compounds to have hypoglycemic and antidiabetic properties. In streptozotocin induced diabetic rats, Zhang et al. (2006) showed hypoglycemic activity of various mushrooms like *Cordyceps militaris*, *Cordyceps sinensis*, *Tricholoma mongolicum,* and *Omphalia lapidescens*. Thus, this chapter deals with some medicinal fungi which are having the natural pharmacological compounds of some important fungi such as *Ganoderma* sp., *Inonotus* sp., *Cordyceps* sp., *Phellinus* sp., and *Xylaria* sp.

18.2 Medicinal Important Fungi

18.2.1 *Ganoderma lucidum*

G. lucidum is one of the bracket fungi which grow on the rotting wood of tree. In China, *G. lucidum* is called as Lingzhi, and in Japan it is called as Reishi. Lingzhi is one of the traditional Chinese medicinal mushrooms known for more than 2000 years (Kladar et al. 2016). *G. lucidum* is known to contain various bioactive molecules, such as polysaccharides, triterpenoids, nucleotides, fatty acids, glycoproteins, sterols, steroids, proteins, and peptides (Wachtel-Galor et al. 2011; Batra et al. 2013). As polysaccharide is main active component, the study of its characteristics, composition, and molecular weight is important in drug designing. Therefore, similar studies have been performing in case of *G. lucidum* for understanding its composition and characteristics. The study reveals that most of the polysaccharides are heterosaccharides with different combinations of sugars and (1 → 3)-, (1 → 4)-, (1 → 6)- α, β-glucans are identified from *G. lucidum* (Wasser 2002; Nie et al. 2013). The antitumor and immunomodulatory effects of the mushrooms are enhanced by polysaccharides with are branched (1 → 3, 1 → 6)-β-D-glucans. These branched polysaccharides bind to β-glucan specific receptors on innate immune cells, namely monocytes or macrophages, which later on might result in activation of adaptive immunity (Akramiene et al. 2007; Rop et al. 2009). These pharmacological active polysaccharides have various properties such as anticancer, antidiabetic, anti-hepatotoxic, and immunomodulatory properties (Boh 2013; Boh et al. 2007; Paterson 2006). Apart from *G. lucidum,* polysaccharides from other *Ganoderma* species are reported to have different types of bioactive molecules and their activities are given in Table 18.1.

Another important pharmacological compound from *Ganoderma* species is triterpenoids which possess anticancerous, antidiabetic, antiviral, antiandrogenic, and anti-inflammatory activities (El-Mekkawy et al. 1998; Min et al. 1998; Wu et al. 2013; Liu et al. 2006; Akihisa et al. 2007; Ko et al. 2008; Fatmawati et al. 2011; Zhao et al. 2015; Zhu et al. 2015). Polysaccharides and triterpenoids from *G. lucidum* are known to induce carcinogen detoxifying phase II-metabolizing enzymes like glutathione transferase, NAD(P)H:quinine reductase, and uridine diphosphate (UDP)-glucuronosyl transferases leading to enhanced immunomodulatory effects (Casson et al. 2003; Kim et al. 1999).

Triterpenes from *G. lucidum* are known to lower the expression levels of antiapoptotic protein and increase the expression levels of proapoptotic protein like B cell lymphoma-2 (Bcl-2) protein resulting in induction of programmed cell death in prostate cancer cells (Ibrado et al. 1996; Lebedeva et al. 2000; Qu et al. 2017). One of the triterpenoids present in *Ganoderma* is Ganoderic acid A, which has been shown to possess anticancerous activity in various types of cancers like osteosarcoma, lymphoma, meningioma, and breast cells by inducing apoptosis (Jiang et al. 2008; Das et al. 2015; Shao et al. 2015; Radwan et al. 2015). Satria et al. (2018) reported a new lanostane-type triterpenoids from the *Ganoderma lingzhi* known as lucidumol D(1) which showed selective antiproliferative and cytotoxic

Table 18.1 Different compound and their biological activities from various medicinal mushrooms

Name of mushroom	Compound name	Activity	Reference
G. applanatum	Fucogalactan and mannofucogalactan	Antitumor	Usui et al. (1981)
G. applanatum	F-I-1a, F-I-1a1β, and F-I-1a2-β	Antitumor	Usui et al. (1983)
G. japonicum	Glucan	Antitumor	Ukai et al. (1983)
G. lucidum	Arabinoxyloglucan	–	Miyazaki (1982)
G. lucidum	Exo-polymer	Hepatoprotective	Song et al. (1998)
G. lucidum	*G. lucidum* polysaccharides (GLP)	Antigenotoxic and antitumor	Kim et al. (1999)
G. lucidum	Fraction A, B, C, D, and E	Immune stimulatory	Habijanic et al. (2001)
G. lucidum	GLE and GLB	Antitumor	Lin (2001)
G. lucidum	GLP	Antitumor	Lee et al. (2001)
G. lucidum	GLP	Protection of macrophages and other cell organelle	You and Lin (2002)
G. lucidum	GLP	Antiulcerogenic	Gao et al. (2002)
G. lucidum	GLP	Antiapoptotic on neutrophils	Hsu et al. (2002)
G. lucidum	GLP	Antitumor and antiangiogenic	Cao and Lin (2004)
G. lucidum	GLP	Neuro protective	Zhao et al. (2004)
G. lucidum	Glycoprotein	Immuno-modulating, antitumor	Wang et al. (2002)
G. lucidum	GLIS	Immune stimulatory	Zhang et al. (2002b)
G. lucidum (broth and basidiocarp)	CW-I, CW-II, HW-I, HW-II, CA –I, CA-II, HA-I, and HA-II	Antitumor	Sone et al. (1985)
G. lucidum (mycelia)	GLP	Hepatoprotective	Zhang et al. (2002a)
G. lucidum (spores)	GLP	Immunostimulant	Bao et al. (2001a)
G. lucidum (spores)	GLP	Immunostimulant	Bao et al. (2002)
G. lucidum (spores)	SP and SP1	Immune stimulatory	Bao et al. (2001b)
G. tsugae (mycelia)	GM_3	–	Peng and Zhang (2003)

(continued)

Table 18.1 (continued)

Name of mushroom	Compound name	Activity	Reference
G. tsugae	EPF1 and EPF2	Antitumor	Peng et al. (2003)
C. sinensis	Glucan	Immunostimulatory	Wang et al. (2017)
C. militaris	Cordycepin	Antitumor and antimetastatic	Jin et al. (2018)
C. cicadae	Polysaccharides	Antioxidative	Olatunji et al. (2016a, b)
P. igniarius	Polyphenols	Antidiabetic	Zheng et al. (2018)
P. linteus	Polysaccharide	Antidiabetic	Kim et al. (2010)
P. igniarius	Methanolic extract	Antioxidant	Liu et al. (2014)
I. obliquus	Betulin, betulinic acid, and inotodiol	Cytotoxicity	Géry et al. (2018)
I. obliquus	Polysaccharide	Antimitotic	Burczyk et al. (1996)
I. obliquus	Polysaccharide	Antitumor and immunomodulatory activity	Staniszewska et al. (2017)

activity against five cell lines, namely human colorectal carcinoma HCT-116 cells, human colorectal carcinoma Caco-2 cells, human breast cancer MCF-7 cells, human cervical cancer HeLa cells, and normal colon epithelial CCD-841 cell line.

Ganoderma species are known to have many secondary compounds. In a study, total 431 secondary compounds were isolated from *Ganoderma* species and out of these 240 were isolated from *G. lucidum* which includes ganoderic acid, lanostanes, lucidenic acid, meroterpenoid, steroid, benzofuran, etc. (Baby et al. 2015). More than 100 meroterpenoids were isolated from *Ganoderma* species are generated in shikimic acid and mevalonic acid biogenetical pathway (Chen et al. 2017). Luo et al. (2016) reported another bioactive miscellaneous meroterpenoids compound isolated from *Ganoderma applanatum* which was found to inhibit COX-2 enzyme responsible for inflammation and pain. Another meroterpenoid (±)-ganoapplanin A (1) isolated from *G. applanatum* have inhibitory activities on T-type voltage-gated calcium channels (Li et al. 2016). Tibetan mushroom *G. leucocontextum* extracted (±)-Ganodilactone meroterpenoid showed pancreatic lipase inhibitory activities (Chen et al. 2016).

18.2.2 *Inonotus obliquus*

According to *Index Fungorum* the systematics position of Chaga mushroom is *Inonotus obliquus* (Fr.) Pilát, *Inonotus,* Family: *Hymenochaetaceae,* Order: *Hymenochaetales, Incertae sedis,* Class: *Agaricomycetes,* Division: *Agaricomycotina,* subphylum*: Basidiomycota, Fungi.* The *I. obliquus* has been used to treat various diseases since sixteenth century in most of the European countries (Cui et al. 2005) and extracts of *I. obliquus* have been used in various countries for their favorable effects on lipid metabolism and cardiac function, and also for antibacterial, anti-inflammatory, antioxidant, and antitumor activities (Shashkina et al. 2006). Fruiting bodies of this fungus are rich sources of various bioactive pharmacological compounds, such as polysaccharides (Mizuno et al. 1999; Yiyong et al. 2015), phenolic compounds (In-Kyoung et al. 2007; Zheng et al. 2009), triterpenes (Zheng et al. 2011), ergosterol and ergosterol peroxide (Kang et al. 2015), inotodiol (Deyao et al. 2011), and melanin (Babitskaia et al. 2000).

Polysaccharides extracted from *I. obliquus* have various activities like antioxidant, antidiabetic, anticancerous, and anti-inflammatory (Ma et al. 2012). Polysaccharides from the fruiting body and the liquid culture from *I. obliquus* suppressed the hydrogen peroxide-induced oxidative damage in RINm5F pancreatic β-cells resulting in prevention of diabetes (Sim et al. 2016). *I. obliquus* polysaccharides induce glucolipotoxicity-induced renal fibrosis in diabetic nephropathy mice, through inhibition of NF-κB/TGF-β1 signaling pathway (Chou et al. 2016). The polysaccharides play a major role in gastrointestinal digestion process; the degradation of polysaccharides leads to better gastrointestinal digestion. This degradation is correlated with increase in the reducing sugar which was due to breakdown of glycosidic bond (Cong Wang et al. 2018).

Inotodiol is a lanostane triterpenoid isolated from *I. obliquus* sclerotia which inhibits cell proliferation through apoptosis induction by activating caspase-3 in mouse leukemia P388 cells. Inotodiol is an interesting compound and can be used for the development of a novel anticancer drug (Nomura et al. 2008).

Phenolic compound 5 isolated from *I. obliquus* had significantly higher free radical scavenging activities resulting in protection against DNA damage (Hwang et al. 2016). Yuki et al. (2007) reported seven components isolated from *I. obliquus* fruiting body which have highest potential antioxidant activity. The findings by Arata et al. (2016) reported that intake of the *I. obliquus* extract in mouse model maintains the body temperature by suppressing the tumor formation and also exhibited body weight loss. One of the bioactive compounds extracted from *I. obliquus* is water-soluble melanin complex which have beneficial effects on lipid metabolism through increased fatty acid oxidation. However, it leads to small change in lipogenic gene expression and increased insulin-stimulated glucose uptake. It also activates AMP-activated protein kinase leading to improved insulin-sensitizing activity without the adverse effect of weight gain. Thus, melanin from *I. obliquus* is one of the potential antidiabetic agent (Jung-Han and Chang-Kee 2014).

Ergosterol peroxide isolated from *I. obliquus* acts as an inhibitor of cancer cells as shown by the colony formation and antiproliferative activity in tumor and colon cell lines. This results in induction of apoptosis and reduces the expression of genes like c-Myc protoocogene expressed during cancer cell proliferation, cyclin D1 protein encoded by CCND1 gene, and increase in expression leads to tumor formation and Cdk-8 kinase, a collateral oncogene. These proteins activate cell proliferation of cancerous cells, suggesting that ergosterol peroxide can be used as an anticancerous drug for curing the cancerous and colorectal cancer (Kang et al. 2015).

18.2.3 *Cordyceps*

The genus *Cordyceps* is one of the caterpillar fungi belonging to perithecial Ascomycota classified as a monophyletic group of order Hypocreales and family Clavipitaceae. There are nearly 400 species reported from this fungus. *Cordyceps* is mainly well known in traditional Chinese medicine for their various medicinal properties including anticancer, antioxidant, immunomodulatory, anti-inflammatory, and antimicrobial activities (Tuli et al. 2014; Yue et al. 2013).

Cordyceps sinensis (CS) (Berk.) Sacc. (Clavicipitaceae) is one of the caterpillar fungus growing within the body of larva from the ghost moth *Hepialus americanus* which is used in Chinese medicine in A.D. 1694 (Yue et al. 2008). *C. sinensis* produced various bioactive compounds such as cordycepin, polysaccharides, ergosterol, mannitol, and adenosine (Yue et al. 2013; Tuli et al. 2013). These pharmacological compounds have antitumor, hepatoprotective, inflammatory antioxidant, nephroprotective, and antiapoptotic activities (Zhong et al. 2012; Kuo et al. 2007; Chen et al. 2005; Yamaguchi et al. 1990; Nakamura et al. 2003; Jordan et al. 2008)

Cordyceps militaris: Culture of *C. militaris* produced Fibrinolytic enzyme which has dual functions, first is to hydrolyze all three chains of fibrinogen (α, β and γ). Second, it acts as anticoagulants which degrade clot formation and for prevention of thrombosis. Thus, this enzyme is one of the novel drugs which might be used for prevention of cardiovascular diseases (Liu et al. 2017). Yang et al. (2012) for the first time reported that the *C. militaris* and its mycelium induced DNA disintegration through the p53 signaling leading to p21 activation and Bcl-2/Bax-mediated pathways resulted in apoptosis and autophagy in human glioblastoma cell line. They also found that downregulation of Bcl-2 leads to apoptosis in carcinoma cell lines.

Cordyceps cicadae is one of the entomogenous fungi that parasitizes on larvae of *cicada*. Xiao Lei was first to discover the pharmacological values of this fungi; *C. cicadae* is one of the most ancient medicinal materials in Chinese pharmacist around 1500 years ago, which is earlier than that of *C. sinensis*. Pharmacological studies of *C. cicadae* showed that the compounds like polysaccharides, cordycepic acid, ergosterol, and effective nucleosides (Chu et al. 2015; Wang et al. 2014) have renoprotective, immunoregulatory, and neuroprotective effects and anticancer activities (Chyau et al. 2014; Olatunji et al. 2016a, b). *C. cicadae* isolated sporoderm-broken spore powders are the active constituents and volatile ingredients

which includes nucleosides, cordycepic acid, cordycepin, beauvericin, and myriocin. The sporoderm-broken spore powder has antineoplastic activity in human lung cancer cells (Sun et al. 2017). Lo et al. (2004) recorded the *Cordyceps* sp. fruit body and carcass effect on antihyperglycemic activity observed in diabetic rats for 26 days. The diabetic rats administered with *Cordyceps* showed higher blood glucose response in oral glucose tolerance, and significantly lower serum fructosamine concentration was observed. Some *Cordyceps* species are reported to have different types of bioactive molecules and their activities are given in Table 18.1.

18.2.4 *Phellinus*

Phellinus belongs to family Hymenochaetaceae. Many species are annual to perennial and some of them causes white rot diseases. Fruit bodies mainly yellowish to rusty brown to gray to black grows on wood. Several species of *Phellinus* are having pharmacologically important bioactive metabolites like polysaccharides, phenols, flavonoids, etc. which are used traditionally for the treatment of different diseases such as cancer, diabetes, and hepatitis allergy inflammation.

Phellinus linteus extract inhibits the proliferation as well as colony formation of human breast cancer cells by upregulation of $p27^{Kip1}$ expression arresting the cell cycle at S phase (Sliva et al. 2008). Polysaccharides from *P. igniarius* have increased serum immune cytokines of interleukin-2, interleukin-12, and interferon-γ, ultimately activating immune system, which may be the antitumor potential mechanism (Gao et al. 2017). Hwang et al. (2005) extracted the exopolysaccharides from the liquid culture of medicinal mushroom *P. baumii* which mainly consists of heteropolysaccharides and proteoglycans and studied their hypoglycemic activity on streptozotocin-induced diabetic rats for 14 days. Kim et al. (2001) reported that by using the *P. linteus* mushroom the plasma glucose, triglycerides, and cholesterol levels decreased by 49%, 32%, and 28% and aspartate aminotransferase activity was significantly reduced. There are numerous protein markers for the diagnosis of diabetes mellitus. Hwang et al. (2007) reported hypoglycemic activity of exopolysaccharides (EPS) produced from submerged culture of *P. baumii* in diabetic rat and also explore the application of proteomics for mining biomarkers of diabetes. *P. linteus* grown on brown rice supplemented to liver-damaged rat showed suppressed liver damage induced by carbon tetrachloride (Jeon et al. 2003). Peroxidation products (10%), catalase (55%), and superoxide dismutase (39%) expression decreased by CCl_4 and it significantly recovered when administered with extracts. Yuhong et al. (2018) reported that a sulfated polysaccharide from *P. ribis* suppressed the cell proliferation, migration, and tube formation which leads to inhibiting the angiogenic-associated diseases. Some *Phellinus* species are reported to have different types of bioactive molecules and their activities are given in Table 18.1.

18.2.5 *Xylaria*

Xylaria is one of the largest genus of wood rotting and soil fungi belonging to a family Xylariaceae of sordariomycetes. Various bioactive metabolites produced by species of *Xylaria* possess various biological activities (Pittayakhajonwut et al. 2005).

X. nigripes belonging to Xylariaceae from Ascomycota has various medicinal properties which include neuroprotection and anti-neuroinflammatory; the compound isolated from this fungi is used in traditional Chinese medicine (Xiong et al. 2016). Some other bioactive compounds have various activities like hepatoprotective, amnesia, neurosis, anemia, women's menstrual disorder, and insomnia (Song et al. 2011; Li et al. 2015), antioxidant and antiradical activity (Ko et al. 2009; Ma et al. 2013), anti-inflammatory activity (Ko et al. 2011), antidepression (Peng et al. 2015), and serotonin-related hypoglycemic activity (Chen et al. 2015). From fermented broth of *X. nigripes* six new eremophilane-type sesquiterpenes are extracted, namely, nigriterpenes, phenolic compound, and fomannoxin alcohols effective against insomnia and depression disorders (Chang et al. 2017).

Chen et al. (2018) reported seven new isoprenyl phenolic ethers from fermented broth of *X. fimbriata*; out of seven, 1, 5, and 7 compounds play a role in anti-inflammatory activity. Total 10 compounds were isolated from *X.*cf. *cubensis*, out of which Tryptoquivaline L, Fiscalin C, Cytochalasin D, and Ergosterol peroxide showed cytotoxicity against lung cancer, and compound chevalone C and helvolic acid showed antimalarial activity (Sawadsitang et al. 2015).

Three isopimarane diterpene glycosides isolated from *X. polymorpha* showed anticancerous potential against various cancers like human prostrate adenocarcinoma, human promyelocytic leukemia, and human cervical carcinoma (Shiono et al. 2009). Some *Innonotus* species are reported to have different types of bioactive molecules and their activities are given in Table 18.1.

18.3 Conclusion

The species *Ganoderma, Phellinus, Innonotus, Cordyceps,* and *Xylaria* are produced various medicinal compounds such as polysaccharides, triterpenes, phenolics, enzymes, and meroterpenoids. These compounds from medicinal fungi have variety of pharmaceutical importance such as antitumor, antimicrobial, anti-hepatotoxic, hypocholesteromic, antioxidative, antidiabetic, immunomodulatory, and anti-inflammatory activities. Thus, there is need to use of these medicinal fungi as a natural source which does not have any side effect on human body. There is also need to explore the knowledge of importance of these medicinal fungi which may improve their applications in the food and medicinal industry. There is need to isolate and identify the bioactive metabolites from these medicinal fungi for better understanding of its pharmacological properties. There is need for cultivation of

these medicinal fungi under greenhouse or in laboratory condition, so that we get more quantity of natural pharmaceutical product from their fruiting body.

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Biochemical Aspects of Syngas Fermentation

19

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Abstract

In the current scenario, the world is facing a shortage of fuels, and in the future, the existing reservoirs of fuels will be exhausted; this challenging situation could be solved by the method of syngas fermentation. Syngas is a mixed composition of gases including carbon monoxide, hydrogen and a lesser amount of carbon dioxide. Mostly, syngas synthesis is performed by acetogenic bacteria. These bacteria utilizes the Wood–Ljungdahl pathway for fermentation to take place. Syngas fermentation of waste biomass has led to the development of biofuels rich in energy and valuable chemicals. Biomass used as a substrate includes municipal waste, crops, chemical wastes, coal, lignin, natural gas and wood. Firstly, biomass gets transformed into carbon dioxide and hydrogen via gasification, these products formed act as substrate for syngas fermentation which is utilized by acetogenic bacteria to produce hydrocarbon-rich compounds. Bacteria used are genetically modified and the reactors are optimized for the scale-up studies for maximizing the yield. This chapter covered the important biochemical aspects of

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syngas fermentation and advantages, biochemical pathway and the microorganisms involved in syngas fermentation. The type of bioreactors used for syngas production, challenges faced during syngas fermentation and future perspectives have been included.

Keywords

Biomass · Acetogenic bacteria · Gasification · Syngas fermentation · Wood–Ljungdahl pathway · Biofuels

19.1 Introduction

Ever-increasing world population has led to an increase in global energy demand. The existing energy reserves are not sufficient to fulfil the global energy demands and due to its carbon emission has affected climate leading to global warming. Hence, there was a need for the development of the sustainable and greener synthesis of energy (Fig. 19.1).

Discovery leads to a generation of biofuels in the form of biohydrogen, bioethanol and biobutanol from renewable sources like corn, sugarcane, wheat and many other food crops. First-generation biofuel increased total demand for agricultural land for production of biofuel producing crops which increased the cost, reduction in the supply of food to the population, carbon emission and higher nitrous oxide emission due to excess use of fertilizers (Daniell et al. 2012). Due to the

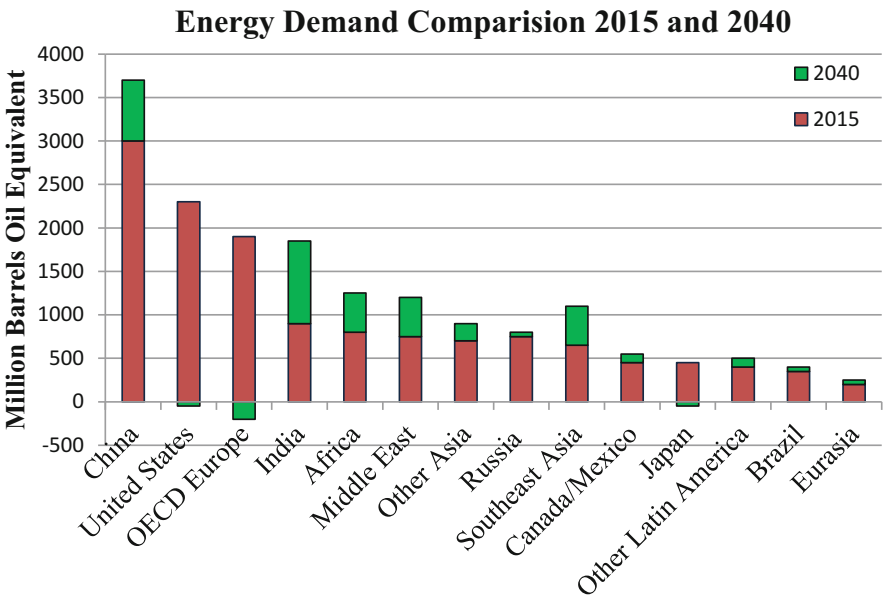

Fig. 19.1 Global energy demand

drawbacks of first-generation biofuel second-generation biofuel came into existence which uses lignocellulosic biomass for production of biofuels as it is not dependent on food or feed and is considered as a renewable feedstock (Daniell et al. 2012; Munasinghe and Khanal 2010).

Lignocellulosic biomass used is mostly formed during agricultural processes as waste (e.g. barley and rice straws, wheat, rice corn stover), energy-producing crops (e.g. poplar, switch grass, miscanthus) and agricultural processing by-products (e.g. sugarcane bagasse, seed cake, corn fibre) for production of biofuel (Munasinghe and Khanal 2010).

Biochemical and thermochemical pathways are the two most important. Researcher too mention worthy approaches for biofuel production from lignocellulosic biomass. In the biochemical pathway, the lignocellulosic material is pre-treated with acid or heat to increase the enzyme activity of pre-treated biomass. Fermentable sugars are obtained by treatment through enzyme hydrolysis which is fermented further to obtain ethanol and by-products (Sun and Cheng 2002). Drawbacks of the biochemical pathway include costly enzyme, high heat treatment, sugar degradation and formation of inhibitory compounds (García-Aparicio et al. 2006). The thermochemical pathway involves gasification of synthesis gas and converting it to biofuel through chemical catalyst process called as Fischer–Tropsch (FT) or by microbial catalyst process called syngas fermentation (Daniell et al. 2012). The drawbacks of using chemical catalyst are high temperature and pressure, toxic gases, and maintenance of constant feed fed composition (Phillips et al. 1994; Vega et al. 1990; Worden et al. 1991).

Syngas fermentation is the process which involves microbes as the catalyst for acetic acid, ethanol, 2,3 butanediol, butyric acid, and butanol formation from lignocellulosic biomass (Daniell et al. 2012; Henstra et al. 2007; Bengelsdorf et al. 2013). Such fermenting bacteria are called as acetogenic bacteria. Microbes used are *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Acetobacterium woodii*, *Clostridium carboxidivorans* and *Peptostreptococcus productus* are able to ferment syngas into liquid fuel (Henstra et al. 2007; Heiskanen et al. 2007). Syngas fermentation is advantageous when compared with biochemical and FT process because of the following reasons: (a) costly enzymes and complex pre-treatment is absent; (b) independent of H_2:CO ratio; (c) metal poisoning absent; (d) highly specific biocatalysts; (e) higher temperature generation of syngas maintains aseptic conditions; and (f) maintenance of optimum conditions of bioreactor (Heiskanen et al. 2007; Bredwell et al. 1999; Brown and Brown 2003).

Commercialization of syngas fermentation had two limitations, i.e. poor solubility of gaseous substrates in the liquid phase and low productivity; these limitations were solved with the help of bioreactor designing (Munasinghe and Khanal 2010). Bioreactor helped in achieving higher product concentration and higher production rates (Asimakopoulos et al. 2018).

This chapter deals with all the aspects of syngas fermentation, i.e. advantages, biochemical pathway, microorganisms used, bioreactors used, potential products and yield, economics, scale-up, commercialization, challenges faced and future prospectus.

19.1.1 Advantages with Syngas Fermentation

Biofuel is mainly produced from gas fermentation, also known as syngas fermentation, as the whole process is based on microbes. First-generation fuels are such as petrol and diesel coal. Combustion of these fuels produces high amount of toxic material which is harmful to nature; gas fermentation has undeniable advantages over the first-generation biofuels technology; it uses both food and non-food biomass with ample amount of feedstock available to produce sustainable volumes of biofuels. Moreover, gas fermentation has numerous advantages when compared to different types of second-generation approach in terms of feedstock, raw material, and production in terms of cost.

The conversion of syngas into liquid hydrocarbon using Fischer–Tropsch (FT) process is done using some types of a metal catalyst such as iron and cobalt thermodynamically; similar to syngas fermentation the process starts with gasification of biomass into synthetic gas. This gas is highly optimized and cleaned by an energy intense water-gas shift step. Now, these treated synthetic gases are then transformed into hydrocarbon through the FT process to produce liquid fuels. It has been shown by some published studies that gas fermentation for ethanol production has greater fuel yield (overall efficiency is 57%) and energy efficient when compared with the FT process (overall efficiency is 45%). It can also be included that ethanol that is produced from biomass through the process of gas fermentation has shown lower emission of carbon and a higher rate of conversion of carbon into fuel when compared with other FT processes (Daniell et al. 2012; Munasinghe and Khanal 2010; Abubackar et al. 2011; Dry 2002).

For second-generation biofuel mainly lignocellulosic biomass is used for gas fermentation. From lignocellulosic biomass feedstock-produced biofuel is identified as a sustainable way to recover the growing energy demands as it has higher availability as biomass; lignocellulosic biomass is composed of leftover or the parts of plant after farming or the parts of the plants which cannot be considered under the class of food and it is also low feedstock cost. During the gasification process, CO and H_2 are formed in the gaseous form from lignocellulosic biomass. Microbial syngas fermentation shows significant advantages over FT synthesis such as syngas quality, gas-liquid mass transfer limitations and product recovery, and microbial catalyst etc. (Munasinghe and Khanal 2010; Perlack 2005).

According to Oxfam briefing paper 2008 “Biofuel policies are deepening poverty and accelerating climate change” this kind of comment was also shared by Greenpeace and United Nations Food and Agriculture Organization (FAO). Another individual Peter Brabeck-Letmathe, the chairman of NESTLE, claimed in Wall Street Journal interview that “Worldwide about 18% of sugar is being used for the production of biofuel” and also said that poor are losing access to food as a result of which he argued with the politicians to stop the production of biofuels from food products and should use other organic materials for biofuel production.

Moreover, the biofuels cannot be concluded as the solution for the climate crisis as they increase the total demand of the agriculture land. Proportionally there is an increase in the use of nitrogen fertilizers which will lead to increase in nitrous oxide

emission. It came into notice that even if all the supply of carbohydrates is used to produce ethanol, it can only cover 40% of petrol consumption.

Economist are not liking the fact that sugar-based biofuels are inversely proportional to food security and if biofuels are legalized throughout the world it may directly result in an increase in the cost of food material and it will increase agriculture land demand which will lead to deforestation. This theory was proved recently when 30 years' highest price was recorded for sugar 795.40 dollar/ton in February 2011 due to the shortage of crops (Daniell et al. 2012; Naylor et al. 2007; Mitchel 2008; Bailey 2008; Ajanovic 2011).

19.2 Raw Materials for Syngas Fermentation

The most abundant sources of carbohydrate on earth are the lignocellulosic biomass products like sugar, starch, and other oil-based feedstock which are used for the production of biofuel through the process of syngas fermentation, e.g.—in USA—bioethanol is produced using corn starch, Thailand—uses cassava starch as a raw material and Brazil—which hold the first rank in the production of biofuel uses sugarcane as the raw material for the production. Other common raw materials which are used for the production of biofuel through syngas fermentation are soybean, rape, canola seeds palm fruits, etc. Lignocellulosic biomass is generally composed of agri-residue, agri-processing by-products, etc. All those which does not compete with feed and food stock are also considered as renewable raw material for biofuel production through gas fermentation (Munasinghe and Khanal 2010; Demirbas 2007; Nguyen et al. 2007; Schubert 2006).

The syngas output was about 70,817 MW thermal in 2010; this was done using 144 operating plants which are present throughout the world with 412 gasifiers. By 2016, many plants which were under construction were expected to come into force, and if all the operational plants are released to its full capacity as planned, it is said that the worldwide capacity will increase up to 72%. The coal was considered as a dominant feedstock in 2010 which provides about 51% of syngas formation, petroleum stock accounts for 25%, natural gas, 22%, petcoke 1% and other types of biomass waste 0.5% (Daniell et al. 2012; Bengelsdorf et al. 2013; Dahmen et al. 2012).

19.3 Microorganisms

The organisms which are used for fermentation of ethanol are generally divided into two major divisions, these are: mesophilic and thermophilic bacteria.

The mesophilic microorganism is known for fermenting syngas into ethanol and other different types of bioproducts which are useful to men. Mesophilic microorganism generally thrives between the temperature of 37 and 40 °C and pH of about 5.8–6.0, unlike thermophilic microorganism which requires high temperature for the survival between 55 and 80 °C. Bacteria like *C. carboxidivorans*, *C. ljungdahlii*,

Acetobacterium woodii and *Clostridium aceticum* are grouped under mesophilic microbes which are widely used and studied for syngas fermentation. Among these microbes *clostridium ljungdahlii* is mostly used; it is a rod-shaped and gram-positive anaerobic bacteria which has the capability to ferment CO and H_2 into acetic acid and ethanol (Munasinghe and Khanal 2010; Henstra et al. 2007).

In 1932, acetogenesis was first identified when the production of acetic acid took place from H_2 and CO_2 by sewage sludge. Klass Wieringa identified *Clostridium aceticum* demonstrating that acetic acid can be synthesized from H_2 and CO_2 by this pure culture. It has been proved that the acetogenic pathway is a very ancient process and this process has self-optimized and self-regulated itself to ensure that only those species which produce acetyl coenzyme A from small molecules present in nature should survive. Acetyl coenzyme A is an intermediate which at later stages gets used up to yield organic acids and alcohols and synthesize cell mass derived chemicals and most importantly ethanol and acetic acid. Some of the acetogens have the ability to reduce organic acids to alcohol (OH group), most importantly acetic acid to ethanol for biofuel production (Munasinghe and Khanal 2010; Klasson et al. 1993).

Acetogens are mainly anaerobic bacteria; this type of anaerobic bacteria can use the acetyl-CoA pathway as in the following ways:

- It provides a pathway for reductive synthesis of CoA from carbon dioxide.
- It also accepts terminal electron and is also considered as energy conservative process.
- It also provides a pathway for CO_2 fixation during synthesis of cell carbon.

These microbes are also known as omnipresent bacteria which are available in nature and have a strong standpoint in nature as it has a key role to play in the global carbon cycle, e.g. *Acetobacterium* and *Clostridium*. Acetogenic organisms are fermented generally for their commercial use; they yield a high amount of butanol, acetate, ethanol, butyrate and 2,3-butanediol (Daniell et al. 2012; Drake et al. 2006; Drake 1994). *Clostridium ljungdahlii* was the first acetogen which was reported to produce ethanol from syngas; later, *Butyribacterium methylotrophicum* was observed to produce butanol and ethanol from CO; other species of acetogenic alcohol producers are *C. autoethanogenum* and *C. carboxidivorans*, which can synthesize butanol and hexanol. *Alkalibaculum bacchi,* which belongs to a different and new genus and species, produces ethanol. *C. difficile* and *C. sordellii* are acetogenic bacteria which act as pathogens for humans which are used for the syngas fermentation; for this reason, selection of bacteria should be taken care with caution (Worden et al. 1991; Phillips et al. 2017, 2015; Barik et al. 1988; Abrini et al. 1994; Liou et al. 2005; Saxena 2008; Allen et al. 2010; Liu et al. 2012).

The enzyme acetaldehyde dehydrogenase is necessary for converting acetic acid to ethanol which has been isolated from *P. productus*, and *C. carboxidivorans*, *C. ljungdahlii*, *C. autoethanogenum*, *A. woodii. Clostridium formicoaceticum* and *Moorella thermoacetica* act as a biocatalyst and are able to convert syngas into a liquefied form more effectively than that of a chemical catalyst (Phillips et al. 2017; White et al. 1989; Fraisse and Simon 1988).

C. autoethanogenum, *C. ragsdalei* and *C. ljungdahlii* produces 2,3-butanediol and have also been reported to produce trace amounts of amino acid-Valine and leucine along with lactase. Acetogenic bacteria can produce butanol, which is synthesized naturally by *C. carboxidivorans*, *Clostridium drakei*, *C. ragsdalei* and *B. methylotrophicum*. Some of the acetogenic bacteria are known to use carbon monoxide (CO) as a sole carbon source. *Oxobacter pfennigil*, *B. methylotrophicum*, *C. carboxidivorans*, *Clostridium scatologenes*, and *Acetonemalongum* can produce butyrate form syngas; *C. drakei* and *C. difficile* have shown butyrate formation during heterotrophic growth (Bengelsdorf et al. 2013; Köpke et al. 2011). n-Hexanol, n-pentanol, isobutanol, n-butanol and n-propanol were formed from n-caproic, n-valeric, isobutyric, n-butyric acid and propionic acid, respectively, with the help of *C. ljungdahlii.* It has been proposed by LanzaTech recently that butanol is the main fermented end product of *C. ljungdahlii* and *C. autoethanogenum* (Bengelsdorf et al. 2013; Perez et al. 2013).

Ethanol is a direct product which is produced by many acetogens; it is produced either directly from acetyl-CoA in a two-step reaction using acetaldehyde or acetate and its reduction to acetaldehyde. But the production of ethanol has not been able to meet its requirement in the market; for these reasons different strategies have been taken up such as mutagenesis and genetic modification.

One of the major bacteria in ethanol production is *C. ljungdahlii*. This bacteria is heterotrophic in nature, which means it can grow on a range of substrates such as fructose-glucose, etc. Its ideal growth temperature is 37 °C. At 37 °C when the syngas is used as substrate ethanol and acetate was produced by *C. ljungdahlii. C. ragsdalei* or *p11 strain*, *C. autoethanogenum* and *A. bacchi* are also being used for the production of ethanol from syngas (Daniell et al. 2012; Köpke et al. 2011, 2010; Huhnke et al. 2010).

Butanol has been considered as an advanced biofuel, it has a higher density when compared to ethanol and some of its properties match with gasoline. Fermentation of butanol is considered as second largest fermentation process in the world after ethanol. The main component of the substrate for the production of butanol is sugar and starch. Bacterial strains like *C. carboxidivorans* or *p7 strain*, *C. drakei*, *B. methylotrophicum* and *C. scatologenes* acts as biocatalyst from the production of butanol-ethanol-acetate; they have an optimal growth temperature, i.e. 37 °C. The first anaerobe to use CO as sole energy and the carbon source is *B. methylotrophicum*, and in 1991 it also produced butanol (Daniell et al. 2012; Liou et al. 2005; Köpke et al. 2011; Michael et al. 2011; Jones and Woods 1986).

The main product of acetogenic bacteria is acetate; the major microorganisms used for acetate production are *A. woodii*, *M. thermoacetica*, and *C. aceticum*. The first acetogen to be isolated from the soil was *C. aceticum*; its substrate includes H_2, CO, CO_2 and syngas and a range of sugar for producing acetate; its optimal growth temperature is 30°C. *M. thermoacetica* is a thermophilic bacteria; its optimal growth temperature is about 55–60°C and it mainly uses sugar as a substrate. *M. thermoacetica* was also the first acetogen to have its genome sequenced and published which help to improve the understanding of acetogenic metabolism. *A. woodii* is also acetogenic bacteria capable of forming acetate form H_2 and CO,

with optimal growth temperature of 30°C, and like any other acetogens it uses glucose, lactate, etc., *A. woodii* genome has also been sequenced for better productivity. *C. scatologenes* is not considered as acetogens but their primary product is acetate (Daniell et al. 2012; Michael et al. 2011; Sim et al. 2007; Parekh and Cheryan 1991; Demler and Weuster-Botz 2011).

19.3.1 Genetically Engineered Bacteria

The technique of gene modification or molecular engineering in different acetogenic bacteria leads to the production of fuel. Many acetogenic bacteria of genus *Clostridium* have been genetically modified to Exide its limitation in production of ethanol and acetate, etc. *Clostridium* sp. MT683 was genetically modified, which was being done by the inactivation of PTA gene which leads to increased yield in ethanol and acetate production was terminated. Furthermore, using synthetic acetaldehyde dehydrogenase gene from *C. ljungdahlii*, the ethanol production was almost doubled. Another strain *Clostridium* sp. MT683 was genetically modified to obtain acetone-producing biocatalyst called *Clostridium* sp. Mace T113, which is described as to produce only acetone eliminating the production of acetate and ethanol (Bengelsdorf et al. 2013; Tyurin et al. 2012; Berzin and Tyurin 2012; Berzin et al. 2012a, b, c, 2013a, b; Kiriukhin and Tyurin 2013; Tyurin and Kiriukhin 2013).

Recently, metabolic engineering and synthetic biology technique have been applied for the improvement of the robustness, productivity in all the types of fermentation process known to man. These types of fermentation also show a high amount of output such as ethanol-to-acetate ratio of the gas-fermenting organism which has been achieved through random mutagenesis which is combined with high-throughput screening for desired characteristics (Daniell et al. 2012).

Clostridium ljungdahlii and *Clostridium autoethanogenum* are reported to be metabolically engineered to integrate a new way for gas fermentation and production of biofuels butanol, whereas metabolically engineered *C. aceticum* produces chemical acetone. Butanol biosynthesis genes such as *hbd*, *crt*, *thlA* and *adhE* have been introduced in *C. ljungdahlii* from *C. acetobutylicum* (model organism). Genes like *adc*, *clfa*, *ctfB* and *thLA* taken from *C. acetobutylicum* are introduced in *C. aceticum*; this modified *C. aceticum* strain to produce high yield (Daniell et al. 2012; Köpke et al. 2010; Köpke and Liew 2012).

19.4 Biochemical Pathway for Syngas Fermentation

The Wood–Ljungdahl pathway also referred as reductive acetyl-CoA pathway is utilized by all the acetogenic bacteria for reduction of H_2 and CO_2 or CO (Bengelsdorf et al. 2013). Intermediate step Acetyl-CoA is formed by the two branches, i.e. methyl (also called as "Eastern") and carbonyl branch (or "Western") (Daniell et al. 2012; Ragsdale 1997). If sugar is present, it undergoes glycolysis and enters the Wood–Ljungdahl pathway via pyruvate:ferredoxin

oxidoreductase reaction (PFOR) (Bengelsdorf et al. 2013; Ragsdale and Pierce 2008). Additional acetyl-CoA is formed by CO_2 produced via PFOR reaction also formed in glycolysis, which is reduced by Wood–Ljungdahl pathway (Bengelsdorf et al. 2013; Ragsdale and Pierce 2008) (Fig. 19.2).

When CO is present as substrate, it gets oxidized to CO_2 by carbon monoxide dehydrogenase (CODH) and one molecule directly enters into the carbonyl pathway (Bengelsdorf et al. 2013; Drake et al. 1980). CO_2 produced enters into methyl branch and undergoes subsequent reduction to form acetyl-CoA. In methyl branch, CO_2 gets reduced to formate by formate dehydrogenase (FDH). Formate interacts with tetrahydrogen-folate (THF) by formyl-THF synthetase (FTHFS) using adenosine triphosphate (ATP), yielding formyl-THF. Formyl-THF gets reduced to methyl-THF through various THF-dependant enzymes. THF-dependant enzymes are methenyl-THF cyclohydrolase (MTC), methylene-THF dehydrogenase (MTD) and methylene-THF reductase (MTR). Methyl of methyl-THF gets transferred to corrinoid iron-sulphur protein (CoFeS-P) and forms the methyl-corrinoid iron-sulphur protein. Methyl-CoFeS-p forms acetyl-CoA through enzyme acetyl-CoA synthase (ACS). ACS is a tetramer of two α subunit and two β subunits; acetyl-CoA is catalysed by α subunit and CO_2 is reduced to CO by β subunit (Bengelsdorf et al. 2013; Ragsdale et al. 1983; Raybuck et al. 1988; Pezacka and Wood 1984; Grahame 2003) (Table 19.1).

Generally, acetate is the main product formed due to acetogenic bacteria which is formed by conversion of acetyl-CoA by enzymes phosphotransacetylase (PTA) and acetate kinase (ACK), yielding one ATP through substrate-level phosphorylation (Bengelsdorf et al. 2013). Hence, overall ATP gained is zero. Protein gradient is present across the membrane of *Moorella thermoacetica* as they possess cytochromes and menaquinone. Cytochromes and menaquinone are also possessed by *Clostridium aceticum* and *Clostridium formicoaceticum* (Bengelsdorf et al. 2013; Gottwald et al. 1975; Braun 1981). Rnf is membrane-bound complex present in *Acetobacterium woodii*, which transfers an electron to NAD^+ through catalysed oxidation of reduced ferredoxin (Bengelsdorf et al. 2013; Biegel and Müller 2010). Rnf complex performs the function of a proton pump in *C. ljungdahlii* (Bengelsdorf et al. 2013; Biegel and Müller 2010).

CO transforms into CO_2 through oxidation which yields ferredoxin when the substrate is CO or syngas (Bengelsdorf et al. 2013; Shanmugasundaram and Wood 1992). When substrates are H_2 + CO_2, two H_2 is converted to reduced ferredoxin (Fd^{2-}) and reduced nicotinamide adenine dinucleotide (NADH) by an electron bifurcating hydrogenase that operates in *A. woodii* and *Thermotoga maritima* (Bengelsdorf et al. 2013; Zeikus et al. 1980). Bifurcation mechanism gene sequences are present in *C. aceticum*, *C. ljungdahlii*, *E. limosum* and *M. thermoacetica*, but biological evidence shows that expression is absent (Bengelsdorf et al. 2013). Rnf complex produces ion gradient which gets converted to ATP by Na^+ or H^+-dependant ATPases (Bengelsdorf et al. 2013; Müller 2003).

C. ljungdahlii, *C. ragsdalei* and *C. autoethanogenum* grown on steel mill waste gas produce 2,3-butanediol (Bengelsdorf et al. 2013; Köpke et al. 2011). Acetyl-CoA gets transformed to pyruvate and then finally gets converted to

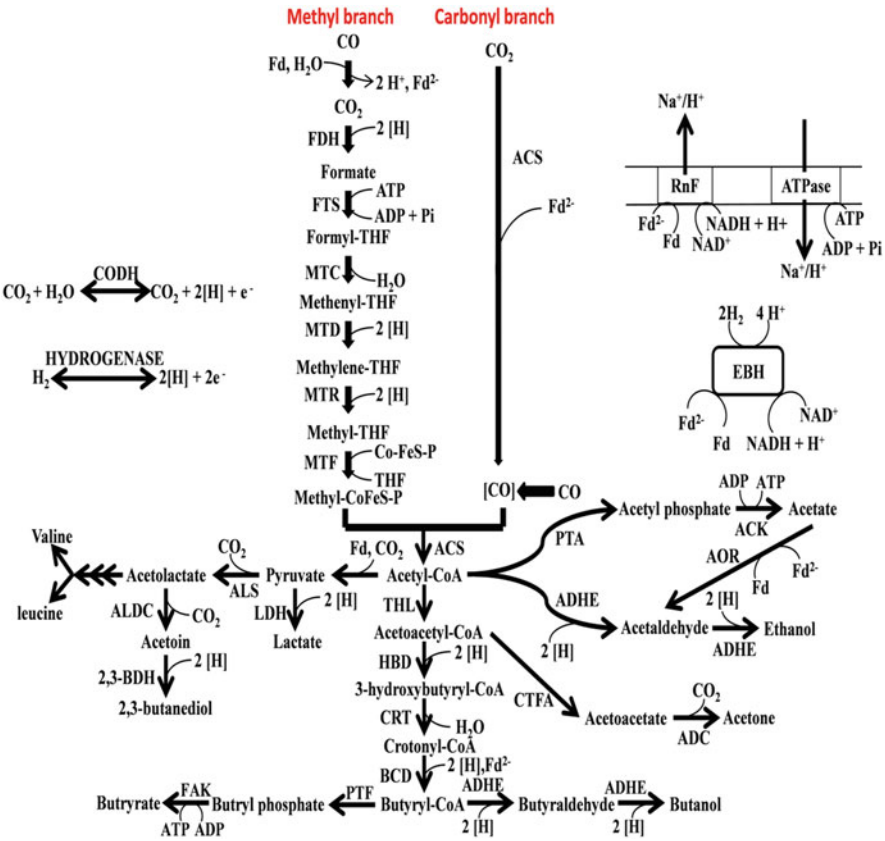

Fig. 19.2 Wood–Ljungdahl pathway. *FDH* formate dehydrogenase, *Fd* oxidized ferredoxin, Fd^{2-} reduced ferredoxin, *THF* tetrahydrofolate, *FTS* formyl-THF synthetase, *ATP* adenosine triphosphate, *ADP* adenosine diphosphate, *MTC* methenyl-THF cyclohydrolase, *MTD* methylene-THF dehydrogenase, *MTR* methylene-THF reductase, *MTF* methyltransferase, *Co-FeS-P* corrinoid iron-sulphur protein, *ACS* CO dehydrogenase/acetyl-CoA synthase, *THL* thiolase, *HBD* 3-hydroxybutyryl-CoA dehydrogenase, *CRT* crotonase, *BCD* butyryl-CoA, *LDH* lactate dehydrogenase, *ALS* acetolactate synthase, *ALDC* acetolactate decarboxylase, *2,3-BDH* 2,3-butanediol dehydrogenase, *PTF* phosphotransferase, *FAK* fatty acid kinase, *ADHE* aldehyde/alcohol dehydrogenase, *CFTA* acetoacetyl-CoA, *ADC* acetone decarboxylase, *PTA* phosphotransacetylase, *ACK* acetate kinase, *AOR* aldehyde oxidoreductase, *EBH* electron-bifurcating hydrogenase, *RnF* complex tetrahydrofolate

2,3-butanediol by enzymes acetolactase synthase, acetolactate decarboxylase and 2,3-butanediol dehydrogenase (2,3-BDH). Valine and leucine are produced by acetogens from acetolactate as a precursor, by gene expression of *ilvC* and *ilvD* genes (Bengelsdorf et al. 2013; Köpke et al. 2011).

Acetaldehyde is formed by reduction of acetyl-CoA which gets converted to ethanol by alcohol dehydrogenase (ADHE) (Bengelsdorf et al. 2013). Ethanol is produced by acetogenic bacteria naturally (Bengelsdorf et al. 2013). Acetogenic

Table 19.1 Enzymes of Wood–Ljungdahl pathway

Enzyme	Reaction	Reference
Carbon monoxide dehydrogenase	$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$	Phillips et al. (2017); Roberts et al. (1992)
Hydrogenase	$H_2 \rightarrow 2H^+ + 2e^-$	Phillips et al. (2017); Ljungdhal (1986)
Ferredoxin oxidoreductase	$Fd_{Rd} \rightarrow Fd_{Ox} + 2e^-$	Phillips et al. (2017); Ragsdale et al. (1987)
Formate dehydrogenase	$CO_2 + NADPH \rightarrow HCOO^- + NADP^+$	Phillips et al. (2017); Yamamoto et al. (1983)
Formate kinase	$HCOO^- + ATP^{4-} + H^+ \rightarrow COOPO_3^- + ADP^{3-}$	Phillips et al. (2017); Mejillano et al. (1989)
Formyl THF synthetase	$HCOOPO_3^- + THF \rightarrow HCOTHF + HPO_4^{2-} + H^+$	Phillips et al. (2017); Sun et al. (1969)
Methenyl THF cyclohydrolase	$HCOTHF + H^+ \rightarrow HC^+THF + H_2O$	Phillips et al. (2017); Ljungdhal (1986)
Methylene THF dehydrogenase	$HC^+THF + NADPH \rightarrow H_2CTHF + NADP^+$	Phillips et al. (2017)
Methylene THF reductase	$H_2CTHF + 2H^+ + 2e^- \rightarrow H_3CTHF$	Phillips et al. (2017); Park et al. (1991)
Methyl transferase	$H_3CTHF + H^+ + [Co^+]E^{2+} \rightarrow THF + H_3C[Co^{3+}]E^+$	Phillips et al. (2017); Ljungdhal (1986)
Corrinoid-iron-Sulphur protein	$[co^+]E^{2+}$	Phillips et al. (2017); Lu et al. (1990)
Acetyl-CoA synthase	$H_3C[Co^{3+}]E^+ + CO + CoASH \rightarrow CH_3COSCoA + [Co^+]E^{2+} + H^+$	Phillips et al. (2017); Park et al. (1991)
Phosphotransacetylase	$CH_3COSCoA + HPO_4^{2-} + H^+ \rightarrow CH_3COOHPO_3^- + CoASH$	Phillips et al. (2017); Drake et al. (1981)
Acetate kinase	$CH_3COOHPO_3^- + ADP^{3-} \rightarrow CH_3COO^- + ATP^{4-} + H^+$	Phillips et al. (2017); Drake et al. (1981)
Aldehyde dehydrogenase	$CH_3COO^- + NADPH + 2H^+ \rightarrow CH_3CHO + H_2O$	Phillips et al. (2017); White et al. (1989)
Alcohol dehydrogenase	$CH_3CHO + NADPH + H^+ \rightarrow CH_3CH_2OH + NADP^+$	Phillips et al. (2017); Fraisse and Simon (1988)

bacteria contain genes which encode for aldehyde:ferredoxin oxidoreductase (AOR-tungsten-dependent enzymes), through which it reduces acetate to acetaldehyde and further gets converted to ethanol (Bengelsdorf et al. 2013; Roy and Adams 2002). *C. carboxidivorans*, *Clostridium drakei*, *C. ragsdalei* and *B. methylotrophicum* are the acetogens which produce butanol naturally (Bengelsdorf et al. 2013; Zeikus et al. 1980).

C. ljungdahlii, *C. ragsdalei* and *C. autoethanogenum* produce lactate in lesser amount (Bengelsdorf et al. 2013; Roy and Adams 2002). Studies of Kita et al. showed that *M. thermoacetica* was able to produce lactate through heterologous expression of lactate dehydrogenase from *Thermoanaerobacter pseudethanolicus* (Bengelsdorf et al. 2013; Kita et al. 2013).

19.5 Bioreactor Design and Configuration for Syngas Fermentation

The most common configuration examined for syngas fermentation is batch mode, continuous mode and semi-continuous mode of operation. It is the most critical deciding factor which must be considered for the growth of microorganisms in a bioreactor. In the batch mode of operation, the bioreactor is fed with the gaseous substrate, liquid nutrient media and microorganisms as the biocatalyst and is let to ferment in a closed system. Whereas, in a continuous mode, the bioreactor is introduced with the gaseous substrate continuously and the fermentation product is withdrawn at the same flow rates as the feed (Munasinghe and Khanal 2010).

Stirred-tanked reactor (STR) operated on continuous mode were the most common bioreactor employed for syngas fermentation. Other commonly used bioreactors are bubble column reactors, fixed bed gasification system, fluidized bed gasification system, hollow fibre membrane reactor, tickle-bed reactor, monolithic biofilm reactor, gas-lift reactor and microbubble dispersion STR.

19.5.1 Continuous Stirred-Tank Reactor (CSTR)

In CSTR, the rate of gaseous substrate feeding into the bioreactor is at the same rate as the removal of a fermentation product from the system (Munasinghe and Khanal 2010; Vega et al. 1990; Klasson et al. 1992). CSTR requires a high level of agitation and mixing for enhanced mass transfer rates which are achieved with the use of gas dispersion baffled impellers. Baffled CSTR can be operated in semi-batch mode, fed-batch mode or with continuous mode. High impeller rotational speed tends to break larger gas bubbles into smaller and finer gas bubbles, thus making the interaction of gaseous substrate more available to the microbial biocatalyst. This also enables longer retention time of gas bubbles in the liquid medium, thereby increasing mass transfer rates.

In a stirred-tank reactor, agitation is a major factor which governs mass transfer rates for uniform conditions throughout the reactor volume. Agitators (one or more) are mounted on a central shaft which is attached to a motor and a gear box enables easy manipulation of the rotational speed. Power number of the impeller is the critical economic parameter which ultimately decides economic feasibility of the process (Yasin et al. 2015).

19.5.2 Bubble Column Reactors

Bubble column reactor is long cylindrical vessels with larger diameter designed mainly for large working volumes without any mechanical parts for agitation or mixing. The reactor is filled with liquid culture media from the top and gaseous substrate is supplied from the bottom of the reactor through spargers. There are two important aspects of the design of this reactor: one is the aspect ratio, i.e. length-to-diameter ratio, and the sparger type which forms the bubbles. The gas in the column spreads due to incremental density differences, thus causing a convective flow current. The merits of using a bubble column reactor are that it provides higher mass transfer rates and lower operational and maintenance costs. It also holds certain demerits, one of which is back-mixing and the other is two or more bubbles fuse together forming a larger gas bubble which shortens the retention time and decreases the mass transfer rates (Munasinghe and Khanal 2010; Datar et al. 2004).

19.5.3 Fixed Bed Gasification System

Fixed bed gasification is an earlier used traditional design for low operation scale applications. It consists of a fixed bed of feedstock over which gasification agent is passed through at an operating temperature of around 1000 °C. Gasification agent is usually passed through updraft direction of airflow or downdraft direction and is thus further classified as updraft gasifiers and downdraft gasifiers.

In updraft gasifier, feed is injected from the top of the reactor and gaseous substrate through the bottom of the reactor and the temperature zone are controlled through air humidification. The top of the bioreactor is at a temperature of about 200–300 °C and here the pre-treated biomass feed is dried. As the temperature increases below this level, the biomass is pyrolysed which causes the release of volatile gases and solid char falls down at the bottom to the air inlet, and combusted at temperature reaching 1000 °C.

Whereas, in downdraft gasifiers, both feedstock and gaseous substrate are introduced from the top of the reactor. The syngas produced from updraft gasifiers were of high energy efficiency, low particle content and high tar content. But in downdraft syngas produced has very low tar content, lower energy efficiency and high particulate content since gases exit the gasifier through the hot zone at a

temperature of 700 °C and as a result partially cracking tar is formed in this process (Daniell et al. 2012; McKendry 2002; Chopra and Jain 2007).

19.5.4 Fluidized Bed Gasification System

Fluidized bed gasifier has a column packed with a bed of finely grained material made of silica sand and is fluidized with flowing stream of gasification medium. The feedstock is added from the top into the unit for the gasification process. This allows the proper mixing of the hot bed material, combusted gaseous substrate and the feedstock and helps achieve homogenous temperature throughout the gasification chamber. Here there are two parts of the unit: one is the gasification chamber with bed material where the pyrolysis occurs and the other is the cyclone separator which separates the ash from the system leaving behind the bed material and char (Daniell et al. 2012). Downdraft gasifiers and fluidized bed gasifiers are the most commonly used system for syngas production from biomass through the gasification process.

19.5.5 Hollow Fibre Membrane

Hollow fibre membrane (HFM) is a gas to the liquid transfer system in which syngas is diffused through the walls of the membrane without forming air bubbles. The membrane serves as the support for the microorganisms growing as a layer of biofilm, thus providing high specific surface area for interaction and fermentation of H_2 and CO into ethanol and acetic acid. The membranes are either submerged in a liquid medium or are connected in series with the reactor. HFM provides several advantages over other bioreactors; it provides high yield, high reaction rate, and increased tolerance to toxic substances in syngas and provides high mass transfer rates under high pressure. One of the major drawbacks of HFM is an increase in the thickness of the biofilm that has a negative effect on microbial growth which is termed as biofouling, thus decreasing its efficiency (Munasinghe and Khanal 2010).

19.5.6 Trickle Bed Reactors

It is a packed bed column operated on the continuous mode in which the liquid culture medium is made to flow down the packed bed onto which biofilm grows. The syngas is made to flow in an upward direction, i.e. counter-currently to the liquid, or downward direction, i.e. concurrently to the liquid. Since the liquid trickles down the pores or gaps of the inert packed material, it is called trickle bed reactors. It requires no mechanical agitation and thus has lower power consumption than stirred-tank reactors (Munasinghe and Khanal 2010; Phillips et al. 2017).

19.5.7 Others

Other most commonly used bioreactors with slight modifications in the already existing form are monolithic biofilm reactor, microbubble dispersion stirred-tank reactor and gas-lift bioreactors. In monolithic biofilm reactor, microbes grow as a biofilm on the bed of carrier media over which gaseous substrate is made to pass through for syngas fermentation and the reactor is operated under atmospheric pressure conditions to make the process economically feasible. Whereas in microbubble dispersion STR, a microbubble sparger is equipped at the bottom of the unit for increasing mass transfer rates. As the size of the bubble decreases, internal pressure increases leading to the increased driving force, and also as the diameter decreases, flux increases causing high mass transfer rates (Munasinghe and Khanal 2010). Gas-lift bioreactor or airlift bioreactor is another reactor configuration in which air is sparged from the bottom of the unit, and depending on the structure it is further divided into an external loop and internal loop reactor. In external loop type, there are two separate conduits, fluid circulates from one conduit to another, while in internal loop type, a central draft separates the chamber into an inner tube and outer tube and fluid circulates from inner tube to outer tube for aeration and mixing.

19.6 Factors Affecting Syngas Fermentation

The biomass derived gas fermentation mediated by microbial biocatalysts depend upon several factors including nutrient media, metal cofactors, types of microbes, the temperature of the reactor, pH of the medium, type and design of bioreactor, mass transfer in the reactor and certain inhibitory compounds released during the fermentation process. So in order to achieve an optimum yield of essential products derived from this process, these factors must be enhanced and optimized (Daniell et al. 2012; Munasinghe and Khanal 2010).

19.6.1 Nutrient Media and Metal Cofactors

The nutrient media provides all essential nutrients for gas-fermenting organisms including minerals, vitamins, trace elements, metal cofactors and reducing agents as well (Daniell et al. 2012; Munasinghe and Khanal 2010). The required end products and the species of a microorganism selected are two important criteria which determine the type of growth media and its constituents. It has been observed that production profile can be improved by transferring the microorganism to nutrient limited media; for example, *C. autoethanogenum* produces improved ethanol yield in a nitrogen-limited media (Daniell et al. 2012; Cotter et al. 2009). *C. ljungdahlii*, *Acetobacterium* and *Thermoanaerobacter ethanolicus* best grow in American Type Culture Collection (ATCC) medium 1754, ATCC medium 1019 and ATCC medium 1190, respectively (Munasinghe and Khanal 2010).

In addition, reducing agents and metal cofactors have been seen to improve microbial productivity. For example, the addition of methyl viologen in the growth medium of *C. ragsdalei* improved ethanol productivity as it increases the concentration of cellular NADH which favours alcohol production through NADH-dependent pathways (Daniell et al. 2012; Girbal et al. 1995). Secondly, for metal cofactors, nickel, a cofactor for enzymes such as CO dehydrogenase and acetyl-CoA synthase, improves CO uptake and improved ethanol productivity in syngas fermentation. Few more important cofactors such as Zn2+, SeO4− and WO4− increase the activity of important metalloenzymes in the Wood–Ljungdahl pathway (Daniell et al. 2012; Ragsdale 2009; Saxena and Tanner 2011).

19.6.2 Type of Microorganisms

Selection of the microbial strain is the most crucial and challenging part for syngas fermentation to obtain the targeted product with the desired yield for commercialization. Mesophilic anaerobic are most frequently used in syngas fermentation which includes organisms such as *Clostridium ljungdahlii*, *C. autoethanogenum*, *Acetobacterium woodii*, *Clostridium carboxidivorans*, *Peptostreptococcus productus* and *Clostridium acetobutylicum* (Daniell et al. 2012; Munasinghe and Khanal 2010). Whereas for commercialization, strain improvement through genetic engineering has proved to be more productive and robust.

19.6.3 Temperature

Temperature optimization is again important as it affects the growth of microbe, substrate utilization by the microbe for fermentation and solubility of gas substrate in the aqueous phase (Daniell et al. 2012; Munasinghe and Khanal 2010). Since most commonly used microorganisms are mesophilic and thermophilic microorganisms, thus 37–40 °C is most favourable growth temperature range for mesophilic and 55–80 °C is for thermophilic. Increased temperature range reduces gas solubility in nutrient medium but it facilitates gas to liquid mass transfer as the viscosity of medium decreases.

19.6.4 pH

The pH is the most important factor for optimum activity of microbes and it also improves the activity of fermenting microbes due to its effect on product composition. It has been studied that decrease in the fermentation pH increases the production of ethanol and other highly reduced products as the organisms shift its metabolism to produce alcohol as primary fermentative as a result of which acetic acid accumulates in the immediate environment. However the optimum pH of the medium depends on the species used for syngas fermentation and it ranges from 5.5

to 7.5. For example, *C. autoethanogenum* has an optimum pH of 4.7 for ethanol production (Daniell et al. 2012; Guo et al. 2010), *Clostridium ljungdahlii* has an optimum pH of 6.0 for acetate as well as ethanol production (Munasinghe and Khanal 2010; Tanner et al. 1993), whereas *Clostridium carboxidivorans* has 6.2 as optimum pH for acetate, ethanol, butyrate and butanol production (Munasinghe and Khanal 2010; Liou et al. 2005).

19.6.5 Bioreactor Configuration

Selection of a bioreactor, its type and design massively depends on its ability to facilitate mass transfer from gas to liquid in syngas fermentation. These gases include CO and H_2 which are transferred into the microbial cell for efficient working. Other than high gas-liquid mass transfer rates, process scale-up, low maintenance, and low operating costs are a few more key parameters for bioreactor design. Bioreactor size is another parameter which majorly depends on the rate of mass transfer for gases having low solubility (Munasinghe and Khanal 2010; Vega et al. 1990). The most common bioreactor configurations used on a commercial scale are CSTRs, bubble column reactors, fixed bed reactor, trickle bed reactors, microbubble sparged reactors and hollow fibre membrane reactors (Daniell et al. 2012; Munasinghe and Khanal 2010).

Mass transfer rates are most commonly described in the form of volumetric mass transfer coefficient per unit power input; studies suggest that designs having highest volumetric mass transfer rates are not necessarily most efficient ones as they require increased power consumption (Daniell et al. 2012; Ungerman and Heindel 2007). In a study of STRs having different impeller designs, it was found that STR with a dual Rushton type impeller design had the highest volumetric mass transfer coefficient but another STR having dual impeller design with axial flow impeller had highest volumetric mass transfer coefficient per unit power input (Daniell et al. 2012; Ungerman and Heindel 2007).

19.6.6 Mass Transfer Rate

In a syngas fermentation reaction, gas-liquid mass transfer is the rate-limiting step (Munasinghe and Khanal 2010; Worden et al. 1991; Dahmen et al. 2012). Mass transfer occurs at four stages—transport of gaseous substrate into a gas-liquid interface, transport into an aqueous phase that is the culture medium, transport of gases into the liquid immediately around the microbial cell and finally diffusion of the gaseous substrate into the microbial cell. Among these four stages, the gas-liquid interface offers major resistance to gaseous substrate diffusion. Also, the diffusion limitations into the culture medium lead to low substrate uptake by microbial cell, thus causing a decrease in productivity. So mass transfer rate as discussed earlier depends on the reactor configuration and the various impellers, agitators and spargers attached to it. It is suggested that to improve mass transfer rates in

CSTRs, agitation speed of impellers must be increased which makes it possible to obtain smaller sized bubbles (Daniell et al. 2012; Bredwell et al. 1999). Bubble column reactor has volumetric higher mass transfer coefficient than STRs mainly because it provides a higher interfacial area. Trickling bed reactors and airlift reactors are also examined to provide efficient mass transfer rates (Daniell et al. 2012; Bredwell et al. 1999).

19.6.7 Inhibitory Compounds

Tar, ash, char particles, ethylene, ethane, acetylene and sulphur, and nitrogen gases are additional constituents present as impurities along with the targeted product which affect the efficiency of the process and inhibit microbial catalysts in syngas fermentation. This inhibitory effect results in low cell growth, cell dormancy, shut down in hydron uptake and decrease in product yield (Munasinghe and Khanal 2010; Ahmed et al. 2006; Bridgwater 1994; Haryanto et al. 2009). Another such compound is a nitrous oxide (NO) which inhibits the activity of hydrogenase enzyme, due to which availability of carbon for product formation is reduced. To overcome such inhibitory effects, 0.0025 μm filter were introduced to remove tar, ashes and other particulate matter away from the syngas (Munasinghe and Khanal 2010; Ahmed and Lewis 2007) and scavenging agents such as sodium hydroxide, potassium permanganate or sodium hypochlorite were introduced to eliminate inhibitory effects of NO (Munasinghe and Khanal 2010; Brogren et al. 1997; Chu et al. 2001). It was also studied that *C. ljungdahlii* was tolerant to inhibitory sulphur gas and was not affected by concentrations of sulphur as high as 5.2% (v/v) (Munasinghe and Khanal 2010; Klasson et al. 1993).

19.7 Potential Products and Its Yield

Acetyl-CoA is the precursor to many different end products produced by acetogenic bacterias; these products include acetate, ethanol, 2,3-butanediol, butanol-hexanol, hydrogen and methane. Acetate and 2,3-butanediol is most commonly used in chemical industries as solvents, whereas ethanol and butanol serve as transportation fuels (Daniell et al. 2012; Bengelsdorf et al. 2013). As known acetic acid lacks the potential to be used as fuel, but there are some organisms which convert acetic acid and other volatile fatty acids into lipids and can be used as biodiesel such as oleaginous yeasts (Daniell et al. 2012; Fei et al. 2011; Jin et al. 2012).

19.7.1 Acetate

The sole end product of many acetogens is acetate or acetic acid. In the first reaction, acetyl-CoA is converted to acetyl phosphate by enzyme phosphotransacetylase (PTA), and in the second step of the reaction, PTA is converted to acetate by enzyme

acetate kinase (AK) also producing one molecule of ATP by substrate-level phosphorylation (Daniell et al. 2012; Bengelsdorf et al. 2013).

Clostridium aceticum has been reported to produce acetic acid from syngas (Daniell et al. 2012; Sim et al. 2007) with 100% conversion and productivity of 1.28 g/L (Daniell et al. 2012; Sim et al. 2007). Other microorganisms include *Morella thermoacetica* which uses Wood and Ljungdahl pathway for acetate production and gives higher yield than *C. aceticum*, 108 g/L produced using sugar as substrate (Daniell et al. 2012), and *Acetobacterium woodii* which utilizes H_2, CO_2 and CO to produce yield up to 44 g/L concentration (Daniell et al. 2012; Demler and Weuster-Botz 2011).

Acetic acid has most of its applications in industrial feedstock produced from feeds supplied by petrochemical source either by acetaldehyde oxidation or by methanol carbonylation (Daniell et al. 2012). It is also used in the production of vinyl acetate and acetic anhydride.

19.7.2 Ethanol

Ethanol is the most desirable product of syngas fermentation due to its wide range of applications. Ethanol production can occur by two reactions, one from acetaldehyde and the other via acetate. In one reaction, acetyl-CoA gets converted to acetaldehyde by enzyme aldehyde dehydrogenase (AHD) and acetaldehyde gets reduced ethanol by enzyme alcohol dehydrogenase (ALD). In the other reaction, acetate produced earlier gets converted to acetaldehyde by aldehyde:ferredoxin oxidoreductase (A:FOR) enzyme and similarly acetaldehyde to ethanol by alcohol dehydrogenase (ALD) (Daniell et al. 2012; Bengelsdorf et al. 2013). *Clostridium ljungdahlii* is ethanol- and acetic acid-producing bacteria by syngas fermentation. It produces mainly acetic acid and ethanol concentrations found in one of the reports were 60 g/L (Munasinghe and Khanal 2010; Vega et al. 1990). When *Clostridium ljungdahlii* is heterotropically grown on glucose, fructose and pyruvate as substrate, then 48 g/L of ethanol concentration is obtained at 37 °C. Other strains of microorganisms with considerable yield are *Clostridium ragsdalei* of ~2 g/L yield at 32–37 °C and *Clostridium autoethanogenum* and *Alkalibaculum bacchi* with nearly 0.32–1.7 g/L yield (Daniell et al. 2012). As stated earlier, it is used as an absolute transportation fuel and is used to supplement gasoline as a fuel blend which thus helps to improve the octane emissions and gives reduced emissions. Ethanol is recovered by successive distillations to obtain improved higher yield (Daniell et al. 2012).

19.7.3 2, 3-Butanediol

Firstly, acetyl-CoA being an intermediate gets converted to pyruvate by enzyme pyruvate:ferredoxin oxidoreductase or pyruvate synthase (P:FOR). Then it subsequently gets reduced to 2,3-butanediol by enzymes acetolactate synthase (ALS),

acetolactate decarboxylase (ALDC) and 2,3-butanediol dehydrogenase (2,3-BDH), respectively (Daniell et al. 2012; Bengelsdorf et al. 2013). *Clostridium ljungdahlii*, *Clostridium autoethanogenum* and *Clostridium ragsdalei* have recently shown to produce 2,3-butanediol from steel mill waste or petrochemical compounds which provides the energy and carbon source for growth. 2,3-Butanediol is a very important precursor for manufacturing of 1,3-butadiene and industrial solvents like methyl ethyl ketone (Daniell et al. 2012; Köpke et al. 2011).

19.7.4 Butanol

Here, two carbon acetyl-CoA is first converted to four carbon butyryl-CoA by enzyme thiolase (THL), 3-hydroxybutyryl-CoA dehydrogenase (HBD), crotonase (CRT) and butyryl-CoA dehydrogenase (BCD) sequentially. Next butyryl-CoA is converted to butyrate by phosphotransbutyrylase (PTB) and butyrate kinase (BK); to butanol by aldehyde:ferredoxin oxidoreductase (A:FOR) and alcohol dehydrogenase (ALD) in the similar steps as ethanol production (Daniell et al. 2012; Bengelsdorf et al. 2013; Phillips et al. 2017).

Clostridium carboxidivorans or P7 strain is well known for naturally producing of ethanol and butanol. *C. carboxidivorans* is reported to yield around 1.60–2.66 g/L of butanol in CSTR at 32–37 °C. It converts CO to fatty acids at high pH with increasing biomass but with decreasing pH biomass formation is not favoured, and at this stage, fatty acids are converted to alcohols (Daniell et al. 2012). Other microorganisms used for butanol production are *Clostridium drakei*, *B. methylotrophicum* and *Alkalibaculum bacchi*. Butanol is considered as a precursor for the synthesis of butyl acrylate and butyl acetate in chemical industries. But it is also considered for use as an advanced biofuel or as a blend in gasoline which enhances its octane number and has high energy density than ethanol (Daniell et al. 2012).

19.7.5 Hydrogen

Syngas production is associated with high microbial toxicity due to high CO contents which result in decreased productivities. It is recently reviewed that microbes can produce biological H_2 from CO by a reaction called biological water-gas shift reaction. In this reaction, CO and H_2O are converted to H_2 and CO_2 by enzyme carbon monoxide dehydrogenase (CMD) under strictly anaerobic conditions.

Microorganisms involved in the production of H_2 are photosynthetic bacterium *Rhodospirillum rubrum* (Younesi et al. 2008), thermophilic bacterium *Carboxydothermus hydrogenoformans* (Zhao et al. 2013) and genetically engineered *Thermococcus onnurineus* NA1 strain which converts 100% CO gas phase to H_2 on comparison with the wild-type strain (Kim et al. 2013).

19.7.6 Methane

Methane (CH_4) was produced by fermenting gas in a continuous process in a CSTR functioning under mesophilic and thermophilic conditions. The inoculum used was a mixed consortium including methanogenic bacteria which reduce carbon sources to methane (Thauer et al. 2008). For 1 mol of methane production a stoichiometry of gas substrate having 4 mol of H_2 and 1 mol of CO_2 is required (4:1 ratio) (Bryant et al. 1968). The reaction was reported to produce a high concentration of methane at mesophilic conditions (37 °C) and atmospheric pressure.

Methane production from syngas was achieved by a triculture system consisting of two methanogenic bacterias *Methanobacterium formicium* and *Methanobacterium barkeri* and a photosynthetic bacterium *Rhodospirillum rubrum* (Klasson et al. 1990; Kimmel et al. 1991).

19.7.7 Others

Apart from these products and its yield, trace amounts of branched chain amino acids like valine and isoleucine are also produced by acetogens such as *Clostridium ljungdahlii*, *C. autoethanogenum* and *C. ragsdalei*, where acetolactate is a precursor and there is found evidence for the expression of genes ilvC and ilvD. As well as lactate is produced in trace amounts by this *Clostridium* sp. from pyruvate by enzyme lactate dehydrogenase (LDH) (García-Aparicio et al. 2006; Bengelsdorf et al. 2013). From syngas fermentation, other proposed potential products include propanol, hexanol, propionic acid, hexanoic acid, acetone, butanediol, isobutanol, fatty acids and biodiesel too (Phillips et al. 2017; Köpke et al. 2011; Liu et al. 2014a, b; Dürre 2016; Hu et al. 2016). There is only one report proposed for biodiesel production from syngas fermentation, wherein two-staged conversion was shown. The first stage was the fixation of CO_2 to acetate by a thermophilic anaerobic microbe *Moorella thermoacetica* and in the second stage acetic acid produced was converted to lipids by genetically modified yeast strain *Yarrowia lipolytica* under aerobic conditions (Klasson et al. 1992; Hu et al. 2016).

19.8 Bottleneck During Syngas Fermentation

Over the past years, gas fermentation is commercialized at a higher rate and is used worldwide for the production of valuable products, mostly chemicals and fuels. Regardless of demand, the main challenge associated with syngas fermentation is scale-up (Daniell et al. 2012). Earlier, the main challenges associated with syngas fermentation were technology and higher cost of biomass production. As the years passed by advancement in the technology lead to a feasible and better process for the production of biomass. Yet there is a need for reliable commercialization process

and contaminants free production of gas (Daniell et al. 2012; Kirkels and Verbong 2011).

When microbes are used as catalyst, they have a higher tolerance level towards a range of impurities. If there is an increase in the concentration of impurities such as tar, nitric oxide and hydrogen sulphide, it affects bacterial growth and product formation (Daniell et al. 2012; Datar et al. 2004; Ahmed et al. 2006; Ahmed and Lewis 2007). Impurities generated during the fermentation process can affect osmolarity, pH, redox potential, etc., leading to enzyme inhibition which directly affects cell viability and hence affecting product yield (Daniell et al. 2012). Hence, to avoid such condition Xu, Tree and Lewis have reviewed all the impurities formed during the fermentation process. The most common impurities are of carbon (tars and CH_4), nitrogen (NO, NH_3, and HCN) and sulphur (SO_2, H_2S, COS) in origin (Daniell et al. 2012; Xu et al. 2011). Tars are the main impurity formed during gasification process (pyrolysis stage). They affect production by assisting cell dormancy (Daniell et al. 2012; Rabou et al. 2009). NO (Nitrogen oxide) concentration is higher than 40 ppm affected metabolism of cells affecting cell growth and inhibiting hydrogenase activity (oxidizing hydrogen for the availability of electron for anaerobic respiration) (Daniell et al. 2012; Ahmed and Lewis 2007). NO can be reduced by sodium hypochlorite or potassium permanganate, sodium hypochlorite (Daniell et al. 2012; Brogren et al. 1997; Chu et al. 2001; Mojtahedi et al. 1995). Ammonia increases osmolarity which leads to cell toxicity, and it also inhibits hydrogenase at a lower concentration (Daniell et al. 2012; Xu et al. 2011). Nitrogen is required for bacterial growth which is originated from ammonia. During the gasification process, nitrogen gets converted to N_2, NH_3, and some NO_2 and HCN, 60–80% nitrogen gets converted to ammonia (Daniell et al. 2012; Mojtahedi et al. 1995). Ammonium ions accumulate in the bioreactor due to higher solubility, and hence low level of ammonia accumulated during synthesis can inhibit microbial catalyst (Daniell et al. 2012; Xu et al. 2011). Conventional method used is wet scrubbing technique to remove ammonia and recently used technique is catalyst hot-gas technology (Daniell et al. 2012; Xu et al. 2010). Hydrogen sulphide when above optimum requirement affects cell growth, and consequently product formation is affected (Daniell et al. 2012; Vega et al. 1990; Grethlein et al. 1992).

Under aerobic conditions, growth of gas-fermenting microbes gets inhibited as oxygen level inhibits enzymes responsible for the reductive acetyl-CoA pathway (Daniell et al. 2012; Ragsdale 1991). Some of the acetogens belonging to genus *Clostridium* species *Clostridium butyricum* and *Clostridium aetobutylicum* have a property to metabolize oxygen and can tolerate aerobic conditions (Daniell et al. 2012; Karnholz et al. 2002; Kawasaki et al. 2005). Superoxide dismutase, NADH-oxidase and rubrerythrin are the enzymes responsible for oxygen metabolism (Daniell et al. 2012; Karnholz et al. 2002; McCord et al. 1971). Karnholz et al. studied *M. thermocetica* and *A. woodii* are acetogens survived in low oxygen level in non-reduced liquid media and also discovered on increasing oxygen level resulted in the prolonged lag phase leading to low product yield (Daniell et al. 2012; Karnholz et al. 2002). The activity of gas-fermenting bacteria under aerobic conditions can be increased by genetic manipulation; one such genetically engineered microbe is

C. acetobutylicum, which can tolerate oxygen level due to glutathione biosynthetic capability (Daniell et al. 2012; Zhu et al. 2011). Oxygen present in the biomass is very less and can be removed by passing syngas over reduced copper or palladium-based catalyst (preferred when acetylene concentration is high) (Daniell et al. 2012).

Wet cleaning, tar cracking, ZnO and active carbon techniques are used for gas cleaning (Daniell et al. 2012). The conventional method, wet cleaning technique involves syngas in contact with water droplets resulting in the water-soluble compound to get dissolved such as ammonia and nitric oxide (Daniell et al. 2012). Tar cracking involves various techniques of thermal cracking, scrubbing with oil or water, plasma cracking, and catalytic cracking (Daniell et al. 2012; Rabou et al. 2009). Hot-gas cleaning technology removes ammonia and tar from syngas more efficiently (Daniell et al. 2012; Xu et al. 2010). Inorganic impurities and hydrogen sulphide are removed by active carbon and ZnO (Daniell et al. 2012).

In anaerobic fermentation cell concentration achieved is low due to limited ATP but it is desired that most of the carbon needed should go into products rather than biomass. To overcome this issue, media optimization can be done for increased biomass formation, cell recycling or cell immobilization can be done to retain cells (Daniell et al. 2012; Qureshi et al. 2005). Achieving high mass transfer rates for transfer of gas into the aqueous phase is the biggest challenge to date. This mass transfer is necessary so that microbes can access it for bioconversion and thus it is said to be the rate-limiting step (Daniell et al. 2012; Klasson et al. 1992). Product synthesis requires a considerable amount of CO_2 and H_2 as the carbon and energy source but CO_2 and H_2 are relatively insoluble which makes this a rate-limiting step (Daniell et al. 2012). Next challenge is risk associated with biocatalyst including bacteriophage infection and poisoning by oxygen or other contaminants. Bacteriophage infection is a major threat to microbial productivity as the symptoms mostly remain undetected. But few symptoms include reduced fermentation rates, reduces yield, reduced cell growth and changes in the cell morphology and population (Daniell et al. 2012; Jones et al. 2000). Although gas fermentation is not much prone to bacteriophage infection probably because of extreme heat causes thermal inactivation a gasification step. Also, fermentation process occurs on minimal media rather than high sugary or plant extract. This infection could be economically very damaging at a commercial stage of the process, potentially requiring an entire plant to be decontaminated and sterilized before the net batch can be carried.

19.9 Conclusion

The production of biomass derived renewable fuels and chemicals would reduce economic dependence on fossil oil and greenhouse gas emissions. In this realm, the fermentation of syngas is a versatile process for fuel and organic chemical products. Syngas (CO, H_2 and CO_2) components are consumed in Wood–Ljungdahl pathway by autotrophic bacteria for the production of volatile fatty acids and alcohol. Inclusion of low cost substrate for microbial medium, improved gas-liquid mass transfer and productivity, developing strategies to produce high value-added

chemicals are key to lessen overall production cost and increasing the feasibility of syngas fermentation towards commercialization. Improvement in the design of efficient reactor, genetically engineered bacteria and fermentation media and process control have been shown to enhance product formation and reduce production cost. Further development is essential in fundamental and applied research areas for this biological gas conversion processes in collaboration with petroleum- and agriculture-based industries.

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Marine Actinobacteria: New Horizons in Bioremediation

20

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Abstract

Pollution of marine environment affects the biosphere leading to severe consequences. Bioremediation of marine pollutants mediated by actinomycetes is viable and a low cost preposition. Marine actinomycetes, in consortia and axenic culture, are highly effective means of degradation of pollutants like petroleum hydrocarbons (PHCs), oil spills, heavy metals, pesticides, and plastics. The bioremediation strategies adapted by the actinomycetes include degradation, biostimulation, and bioaugmentation. The actinomycetes genera involved are *Rhodococcus*, *Streptomyces*, *Nocardiopsis*, *Actinopolyspora*, *Gordonia*, *Dietzia*, *Janibacter*, and *Micromonospora*. Many environmental factors, such as pH, availability of the pollutants, aerobic environment, toxicity of the pollutant, and microbial diversity affect the bioremediation. There are many chemical, physical, and biological methods which can accelerate the efficiency of the bioremediation.

Keywords

Marine actinomycetes · Bioaugmentation · Biostimulation · Bioremediation

20.1 Introduction

Marine environment is the largest habitat on earth as the ocean cover up the 70% of earth surface (Subramani and Aalbersberg 2012; Behera and Prasad 2020). Among the three domain of life, Bacteria, Archaea, and Eukarya dominate with respect to total biomass and metabolic activity in marine environment. Microorganism plays an essential role in the bio-geological cycles and decomposition of organic matters.

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Among the diverse marine organisms, Actinobacteria are considered as ecologically and biotechnologically important prokaryotes.

Actinobacteria are known to produce more than 45% of bioactive metabolites of microbial origin. Genus *Streptomyces* solely produces 7600 biologically important secondary metabolites out of more than 10,000 produced from actinomycetes. Bacteria belonging to the phylum Actinobacteria reflect huge diversity with respect to morphology, physiology, and metabolic properties. Among the nearly 70,000 natural compounds of the microbial origin, 29% are represented by actinobacteria. They are highly diverse and well-characterized groups of microorganisms, playing crucial role in soil environment by carbon recycling through decomposition of different organic matters (Anteneh and Franco 2019). They are widely distributed in terrestrial as well as marine ecosystems and represent several phenotypes including spore former, aerobes, anaerobes, and filamentous forms (Lewin et al. 2016; Rathore et al. 2019). They are isolated from marine environment such as ocean sediments (Solano et al. 2009), sea-water (Sheikh et al. 2019), marine sponges (Gandhimathi et al. 2009, Sheikh et al. 2019), marine organic aggregates, and deep sea gas hydrate reservoirs (Lam 2006).

Marine actinobacteria are free living as well as associated with marine organisms. Several genera like *Dietzia*, *Salinispora*, *Streptomyces*, *Rhodococcus*, *Marinispora* and *Aeromicrobium marinum* require brine for their growth as they are isolated from marine environment (Heald et al. 2001 Moran et al. 1995, Mincer et al. 2002, Kwon et al. 2006, Bruns et al. 2003). The marine actinobacteria are potential source of novel natural products applicable in industries, agriculture, and medical fields. Exploitation of marine actinobacteria for novel secondary metabolite is relatively a new venture. The novel metabolites comprise Abyssomicin C, a novel antibiotic produced by *Verrucosispora* strain, salinosporamide A, proteasome inhibitor by *Salinispora*; lajollamycin, lactam antibiotic from *Streptomyces* strain, and Diazepinomicin, an antimicrobial alkaloid from *Micromonospora* (Lam 2006). Marine actinobacteria produce a diverse range of enzymes, such as α-amylase, alkaline proteases, cellulase, chitinase, xylanase, keratinase, L-glutaminase, and α-galactosidase (Sharma and Singh 2016; Thakrar and Singh 2019; Murugan et al. 2007; Ningthoujam et al. 2016; Sanjivkumar et al. 2017; Balagurunathan et al. 2010; Temuujin et al. 2016; Gohel and Singh 2015). Besides the production of antibiotics, enzymes, and other valuable secondary metabolites, the actinomycetes play an important role in biodegradation of various pollutants, such as pesticides, plastics, rubber, heavy metal, and other organic matters (Tseng et al. 2007; Diez 2010).

Marine environments are extremely diverse due to their variation in sea surface temperature, salinity, pH, currents, and wind patterns. Due to constant changes in environment, the marine bacteria are adapted to adverse conditions. Therefore, the Actinobacteria of the marine habitats are potential candidates for the bioremediation of heavy metals, hydrocarbon, recalcitrant, and xenobiotic compounds (Albarracín et al. 2010; Benimeli et al. 2003, Benimeli et al. 2007; Polti et al. 2009, Polti et al. 2014; Vobis 1997).

20.2 Global and Indian Scenario of Actinobacteria-Mediated Remediation

The application of marine actinobacteria for the biodegradation of various natural and synthetic substances is increasingly drawing worldwide attention. Due to industrial activities, high amount of organic compounds and heavy metal waste are released in environment resulting in pollution. Applications of microbial population in waste water treatment have been promoted to develop the ecofriendly approach (Sim et al. 2010; Frigon and Wells 2019). Among various microorganisms used in the treatment, marine actinobacteria have received greater attention due to their self-protection and survival tools that led to the production of unique complex entities (Sigrid et al. 2008; Hozzein et al. 2012). The genera *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, and *Nocardia* are reported for the degradation of petroleum hydrocarbon in aquatic habitats (Atlas 1981). Marine actinomycetes are reported for the degradation of agar, alginates, cellulose, chitin, oil, and other hydrocarbons (Chavan et al. 2013).

Marine *Streptomyces* sp. is reported to play an important role in bioremediation of heavy metals. Biosorption of heavy metals Cu and Zn and degradation of xenobiotic compound, Carbaryl, a commonly used but toxic insecticide, from the effluent by the marine actinobacteria namely *Streptomyces acrimycini* NGP, *Streptomyces albogriseolus* NGP, and *Streptomyces variabilis* NGP are reported from the marine sediments of south Indian coastal region, Tamil Nadu (Selvam and Vishnupriya 2013a, 2013b). In yet another study, biosorption of other heavy metal such as Cr (III) and Cr (VI) by marine *Streptomyces* sp. from the sediments of Bay of Bengal Coast of Puducherry is described (Saurav and Kannabiran 2011). Actinobacteria isolated from Bay of Bengal degrade oil like olive, sunflower, gingelly, diesel, and petrol. They also possess chitinolytic activity for the degradation of chitin of worms, cockroaches, and fungi, and thus can serve as a good candidate for biopesticide (Kulkarni and Bee 2015). Another marine actinobacterium, *Nocardiopsis* sp.13H, of the Order Actinomycetales, was assessed for the absorption of cesium ion under different environmental factors (Sivaperumal et al. 2018). Recently, bioremediation of strontium (Sr^{2+}) ion radionuclide by EPS (Extracellular Polymeric Substances) has been reported by a marine *Streptomyces* sp. CuOff24, isolated from Southeast coast of India (Kamala et al. 2019).

There are some reports from the Indian researcher on the oil-degrading abilities of the marine actinobacteria. *Rhodococcus* sp. isolated from the oil-polluted coastal region near Mumbai is capable to degrade crude oil, with ability to optimally grow in 0.4 M NaCl and tolerating up to1.7 M NaCl. The biodegradation ability of *Rhodococcus* sp. in hydrocarbon-contaminated soil and other aquatic system is significant from bioremediation stand point (Sharma and Pant 2001).

Bioemulsifiers are amphiphilic in nature and can lower the interfacial tension and thus can be used in the formation of microemulsions. They can thus replace or reduce the application of harmful petroleum-based synthetic chemical surfactants in the remediation of crude oil, hydrocarbon, and petroleum oil pollution (Margesin and Schinner 2001a, b; Olivera et al. 2003). Bioemulsifier from marine

actinobacteria are less explored in global context. Marine actinobacteria, namely *Streptomyces* sp. S1, isolated from coastal region of Goa, India, produced bioemulsifier (Kokare et al. 2007). An oil-degrading *Rhodococcus erythropolis* strain 3C-9 capable to produce two types of biosurfactants is reported from seaside soil of the Island of Xiamen, China. The biosurfactants enhanced the solubility of hydrocarbon and degradation rate of hexadecane (Peng et al. 2007).

Many marine actinobacteria associated with different invertebrate hosts have attracted attention during the recent years (Sheikh et al. 2018). Biosurfactant production from sponge-associated marine actinomycetes *Nocardiopsis alba* MSA10 has been reported (Gandhimathi et al. 2009). Similarly, *Nesterenkonia* sp. MSA31 associated with the marine sponge *Fasciospongia cavernosa* from southwest coast of India is recognized for the production of biosurfactant (Kiran et al. 2017). *Rhodococcus* sp. TW53 from Pacific Ocean deep sea has been reported for oil-degrading biosurfactant (Peng et al. 2008).

20.3 Strategies for Bioremediation

20.3.1 Applications of the Defined Mixed Cultures

Bioremediation using a mixed culture or consortia is an attractive approach due to the synergistic metabolism, which increases the efficiency of the removal and/or degradation of hydrocarbon and other pollutants. Consortium of the microbial cultures enhances the bioremediation process, as metabolite, intermediates, and end products of one type of bacteria can be utilized by another one for significant link for degradation. Therefore, efforts have been made for using microbial consortia for bioremediation and wastewater treatment (Brenner et al. 2008). Removal of organochlorine pesticides (OCPs), persistent organic pollutants such as lindane and methoxychlor was improved by using defined consortia of actinobacteria (Fuentes et al. 2011, 2013). A mixture of *Streptomyces* strains are reported for chlordane remediation (Fuentes et al. 2016). In another instance, a consortia with three genera, *Pseudomonas*, *Rhodococcus*, and *Gordonia*, from Patagonia, Argentina, was reported for improved removal of polycyclic aromatic hydrocarbons (PAHs). A combination of five strains of these three genera removed 100% of naphthalene and phenanthrene and also showed the highest pyrene biodegradation activity (Isaac et al. 2015). The actinobacteria are proved to degrade pesticide with the ability to resist heavy metals, providing a viable alternate to remediate co-polluted soils with Cr(VI) and lindane (Saez et al. 2015; Polti et al. 2014). Similarly, removal of lindane and Cr(VI) from contaminated soil was reported by a consortium of *Streptomyces* sp. M7, MC1, A5 and *Amycolatopsis tucumanensis* ABO (Aparicio et al. 2018). Similar to pesticides and PAH removal, bioremediation of heavy metal has also been effectively demonstrated by a mixed culture instead of individual pure culture.

Microorganisms have developed resistance against heavy metal by different strategies, involving extrusion, oxidase, reductase, exopolysaccharide, and synthesis

of metallothioneins. The mixed cultures have gained complementation with each other to enhance resistance properties. Bacteria may interact with the heavy metals in many ways, such as extrusion of metals in nontoxic form, which can further be immobilized by exopolysaccharide produced by another bacterium. Other strategies include chemical modification of the metal by oxidation or reduction, absorption by second bacterium, and biomineralization of heavy metals, in which the strain may depend on rest of the culture for the nutrition requirement. The advantages of using consortium over individual bacterium are significant for bioremediation processes (Brenner et al. 2008).

20.3.2 Cell and Enzyme Immobilization

Immobilization of cell is the process in which the intact cells are localized at certain region of space to limit their free migration, while retaining the catalytic activities for repeated and continuous applications. This technique has been employed in the past few decades for the waste treatment. The application of immobilized cells has certain advantages such as high biomass, high metabolic activity, and cost effectiveness. The immobilization of cell enhances the performance, with increased tolerance to toxic compounds, greater enzyme production, avoidance of the cell wash, and extended reaction time for biotransformation or biochemical reactions (Martins et al. 2013). Polyvinyl alcohol-alginate-based immobilized *Streptomyces griseus* was used for the bioremediation of chromate-contaminated effluents (Poopal and Laxman 2009). *Nocardiopsis* VITSISB isolated from Marina beach, Tamil Nadu, India, was immobilized with calcium alginate for the degradation of engine oil. Immobilization of biosurfactant-producing organism was adjudged as a rapid, non-toxic, inexpensive, and versatile method (Roy et al. 2015).

Among many systems of immobilization, encapsulation has been reported as a successful tool for actinobacteria for a slow release delivery system of mineralization of different pollutants. Vancov et al. (2005) reported that encapsulated *Rhodococcus erythropolis* NI86/21 on alginate beads has potential to reduce atrazine levels in aquatic and terrestrial environments.

Coculture immobilization has also been studied for bioremediation process. For instance, Bazot et al. (2007) have examined the mineralization of diuron (herbicide) employing coculture immobilization of actinobacterium *Arthrobacter* sp. N4 and *Delftia acidovorans* W34. The degradation of diuron was increased three times as compared to free cells due to microenvironment created within the beads leading to enhanced availability of oxygen and substrates.

Recently, immobilization of a protease from a haloalkaliphilic actinobacteria is reported to enhance half-life, stability, and activity of the enzyme (Thakrar and Singh 2019). Similarly, immobilization of tyrosinase of marine *Streptomyces espinosus* strain LK-4 was reported for successful phenol removal from waste water (Roy et al. 2014).

20.3.3 Actinobacterial Biosurfactants and Bioremediation

Merely microorganisms are not only responsible for bioremediation, their derived products also play an important role in the process. The use of products of microbial origin has several advantages over the whole cell. Among the wide range of substances used in bioremediation, surface-active compounds have focused attention as suitable candidates. The surface-active compounds are amphiphilic molecules, categorized as: (1) biosurfactant (low molecular weight), such as lipopeptide, glycolipids, and phospholipids and (2) bioemulsifiers (high molecular weight) such as protein, polysaccharides, lipoproteins, lipopolysaccharides, and others.

Surface-active compounds are highly in demand over synthetic molecules due to their low or non-toxicity and higher biodegradability in environment. They have ability to withstand extreme conditions of temperature, pH, and salinity. Biosurface-active molecules form micelles with pollutant as their hydrophobic part favor the solubility and removal of hydrophobic pollutants such as pesticide. On the other hand, the polar groups of the molecules bind and stabilize the metals in the micelles which can be removed from soil.

Many genera of actinobacteria produce surface-active compounds applicable in various fields. Among them, *Streptomyces* is known to produce many secondary metabolites, including surface-active compounds. A marine *Streptomyces* B3, isolated from the west coast of India was reported to produce biosurfactant which is effective over a wide range of pH, temperature, and salt concentrations (Khopade et al. 2012). Another strain of the same species, *Streptomyces* sp. MAB36 isolated from marine sediment proved a potential biosurfactant producer. The culture conditions for the optimum glycolipid production were optimized and fructose and yeast extract were best carbon and nitrogen sources for the biosynthesis of the biosurfactant (Manivasagan et al. 2014). Different species of *Rhodococcus* are also known to produce glycolipid, which acts as biosurfactant. *Rhodococcus erythropolis* and *Rhodococcus aurantiacus* were found to produce glycolipid (Kokare et al. 2007). Biosurfactant of microbial origin have been widely used in enhanced oil recovery, oil spills control, detoxification, and biodegradation of oil from contaminated area (Rahman and Gakpe 2008).

Some other genera of the actinobacteria, such as *Brachybacterium paraconglomeratum* MSA21 and *Brevibacterium aureum* MSA13, associated with marine sponge are also reported to produce biosurfactants using industrial and agriculture waste. Another sponge-associated marine actinomycetes *Nocardiopsis alba* MSA10 was evaluated for the production of lipopeptide biosurfactant (Gandhimathi et al. 2009). There have been efforts for the large-scale production of surface-active agents using inexpensive and low cost raw material (Kiran et al. 2010a, b, Kiran et al. 2014).

20.3.4 Bioremediation of Organic and Inorganic Pollutants

The remediation of contaminated soil with both organic (petroleum, pesticides, herbicides) and inorganic pollutants (heavy metals) is difficult, as remediation strategies vary from compound to compound. Several studies have been carried out on the bioremediation of mixture of pollutant by bacteria and fungi (Liu et al. 2017). The presence of heavy metal could affect the degradation of organic pollutants thorough the formation of unspecific complex in cell, and thus inhibiting the activity of bacteria involved in degradation. Metal ion binds to the sulfhydryl (-SH) group of enzymes tightly which are essential for microbial metabolism or degradation pathways (Olaniran et al. 2009). There are two mechanisms of bioremediation of co-contaminated site. Firstly, lowering the bioavailability of metal using chelating agents or heat treatment and secondly by biostimulation or bioaugmentation with metal-resistant microorganism.

The bioaugmentation with actinobacteria is reported to degrade pesticide in the presence of heavy metals. For instance, Polti et al. (2014) demonstrated different strategies of bioremediation of soil co-contaminated with both organic (lindane) and inorganic (Cr (VI)) pollutant. Six actinobacterial strains are reported for lindane degradation (*Streptomyces* sp. A2, A5, A11, and M7) and metal transformation (*Streptomyces* sp. MC1 and *Amycolatopsis tucumanensis* DSM 45259), used singly and in consortium. Among these strain, *Streptomyces* sp. M7 studied in detail for various physicochemical parameters affecting bioremediation of Cr (VI) and linden (Aparicio et al. 2015). However, reports on the bioremediation of these pollutants by marine actinobacteria are rather scarce.

20.3.5 Marine Actinomycetes in Bioremediation

Marine actinomycetes are halophilic and alkaliphilic in nature. These microorganisms possess a wide range of adaptation and are known for their ability to produce antibiotics, enzymes, and therapeutic agents (Rathore et al. 2019). The marine actinomycetes are reported for the degradation of oil spills, chlorobenzenes, aliphatic and polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), phenols, n-alkanes, heavy metal compounds, and plastic materials. The predominant genera in the degradation and bioremediation are *Rhodococcus*, *Nocardiopsis*, *Streptomycetes*, *Gordonia*, *Dietzia*, *Actinopolyspora*, *Amycolicicoccus*, *Micrococcus*, *Arthrobacter*, *Salinispora*, *Micromonospora*, *Actinomadura*, *Streptosporangium*, *Streptomonospora*, *Prauserella*, *Pseudonocardia*, *Aeromicrobium*, *Marinactinospora*, and *Microbacterium.* The marine ecosystem is polluted with different categories of pollutants resulting into tedious degradation. The degradation processes depend on the composition of pollutants and environmental conditions of the polluted site. The possible mechanisms for the bioremediation in marine ecosystem include co-metabolism, biostimulation, bioaugmentation, bioventing, and biosparging. The consortia are more efficient compared to an axenic culture in bioremediation.

Rhodococcus genus is an efficient degrader of marine pollutants. Aliphatic compounds are potentially degraded by *Rhodococcus* sp. strain Q15. *Rhodococcus erythropolis* species isolated from an oil-polluted soil is effective degrader of the n-alkanes and iso-alkanes at about 10% of the NaCl concentration. *Rhodococcus* sp. reported by Borzenkov et al. (2006) degrades diesel at 15% of the NaCl concentration. *Rhodococcus* sp. *DB11* and *Rhodococcus erythropolis strain DCL14* is able to degrade Octane (C_8H_{18}) at 6.0% and 2.5% of NaCl concentration, respectively (Plotnikova et al. 2011; de Carvalho and ds Fonseca 2005). *Rhodococcus erythropolis* strain degrades hexadecane ($C_{16}H_{34}$) at 7.5% of NaCl concentration (de Carvalho 2012). *Rhodococcus* sp. *TW53* produces a lipopeptide biosurfactant helping bioremediation in sea environment. *Rhodococcus erythropolis 3C-9* produces glycolipid and trehalose lipid biosurfactant which help the marine actinobacterium in the oil spill cleaning in marine environment (Peng et al. 2007). These biosurfactants increase the bioavailability of those pollutants which are not so soluble in the marine water. *Rhodococcus fascians* species cultivated from the Antarctica produces bioemulsifiers which help in the biodegradation of hydrocarbons at low temperature.

Rhodococcus fascians use hexadecane and biphenyl as carbon source at 4–35 °C with mineralization (Yakimov et al. 1999; Margesin and Schinner 2001a, b; Alegbeleye 2018). *Rhodococcus* possess a wide range of catabolic versatility and its strains are reported to degrade naphthalene using structural genes nar Aa, nar Ab, and nar B (Kulakov et al. 2005; Larkin et al. 2005; Ghosal et al. 2016). These genes are not arranged in the single operon. The *narAa* and *narAb* genes collectively involved in the production of α- and β-subunit of the naphthalene dioxygenase (NDO), a key player in the naphthalene degradation. *Rhodococcus* sp. strain RHA1 is involved in the degradation of aromatic phenyl acetate compound (Navarro-Llorens et al. 2005). *Rhodococcus* sp. NCIM 5126, isolated from the oil-polluted coastal region near Mumbai, is an efficient degrader of the crude oil (Sharma and Pant 2000). The *Rhodococcus ruber* of marine origin is reported to degrade plastic waste up to significant level. This organism can degrade 8% of dry weight of plastic waste within 30 days under in vitro conditions (Andrady 2011). *Rhodococcus erythropolis BL1* and *Rhodococcus pyridinivorans S85-2* isolated from the coastal region can degrade the acetonitrile in higher concentration (6 M) using the enzyme nitrile hydratase (Langdahl et al. 1996; Kohyama et al. 2006). *Rhodococcus* sp. YHLT-2 strain, a moderately halophilic bacterium able to grow with 7% of NaCl, can degrade the short- and long-chain hydrocarbons of the petroleum oil (Ryu et al. 2006). *Rhodococcus rhodochrous* isolated from the marine environment of Kuwait degrade the crude oil hydrocarbons (Sorkhoh et al. 1990).

Nocardiopsis alba MSA10 produces a lipopeptide type of biosurfactant and thus help in bioremediation. Similarly, *Nocardiopsis lucentencis* MSA04 being able to produce a lipopeptide biosurfactant can help in bioremediation of seawater (Kiran et al. 2010a, b). *Nocardiopsis* sp. NCIM 5124 isolated from an oil contaminated marine environment is able to degrade the long-chain hydrocarbons with the coproduction of extracellular protease (Dixit and Pant 2000a, b). The bioaugmentation procedure was adapted for the remediation of hydrocarbons, such

as n-tetradecane, n-hexadecane, n-octadecane and Bombay high crude oil. Ammonium chloride as an inorganic nitrogen source played a vital role in the degradation efficiency of oil and hydrocarbons. *Nocardiopsis aegyptia* isolated from the marine sediments degrades polyesters like poly-hydroxyl butyrate (PHB), involving the extracellular enzyme PHB depolymerase (Ghanem et al. 2005).

Nocardiopsis sp. 13H can adsorb cesium ion on its extracellular polymeric substances (EPS) on the cell surface up to 50 mM of $CsCl_2$ concentration. Therefore, this species can remediate the radioactive nuclei in radioactive polluted environment (Sivaperumal et al. 2018). *Nocardiopsis sp. 13H* can also adsorb radionuclei strontium (Sr^+) up to 30mM concentrations (Sivaperumal et al. 2018). *Nocardiopsis sp. VITSISB* isolated from the marine origin produces biosurfactant (rhamnolipid) which can efficiently degrade engine oil and oil spills in the oceans (Roy et al. 2015).

Streptomyces VITDDK1, *VITDDK2*, and *VITDDK3* of marine origin are involved in the bioremediation of heavy metals like Cd (II) and As (V), respectively (Lakshmipathy and Kannabiran 2010; Lakshmipathy et al. 2010). The mechanism of the bioremediation is still to be understood among these organisms. *Streptomyces VITSVK9* isolated from the Bay of Bengal can remediate Cr (III) and Cr(VI) by biosorption (Saurav and Kannabiran 2011). *Streptomyces A160*, *Streptomyces acrimycini NGP*, *Streptomyces albogriseolus NGP*, and *Streptomyces variabilis NGP* isolated from Bay of Bengal and marine sediments of Tamil Nadu coast (India) can remediate Cu(II) by biosorption (Yadav et al. 2009; Selvam and Vishnupriya 2013a, b). *Streptomyces acrimycini NGP*, *Streptomyces albogriseolus NGP*, and *Streptomyces variabilis NGP* are also known for the bioremediation of Pb (II) by biosorption (Selvam and Vishnupriya 2013a, b). *Streptomyces albiaxialis* can degrade diesel and other hydrocarbons at a wide range of concentrations of 3–30% NaCl. *Streptomyces albidoflavus*, a halotolerant species, can completely mineralize nitrobenzene, phenol, and toluene up to 2 M NaCl concentration (Ai et al. 2008).

Streptomyces sp. TIS6 (6H) remediate Cs^+ radioactive metals up to 30 mM of $CsCl_2$. It can also remediate strontium (Sr^+) radionuclei up to 10 mM of $SrCl_2$. *Micromonospora chalcea* isolated from saline soil degrades carbofuran pesticide, where it is used as sole carbon source in minimal medium with co-metabolism. The co-metabolism plays key roles in the degradation (Jayabarath et al. 2010). *Micromonospora sp. J3S17* tolerates and efficiently degrades $CsCl_2$ and $SrCl_2$ up to 10 and 30 mM concentrations, respectively.

Arthrobacter sp. *SN17* degrades octane (C_8H_{18}), naphthalene, biphenyl, salicylate, o-phthalate, gentisate, and phenanthrene at the 3–6% of NaCl concentration in marine water (Plotnikova et al. 2011). *Arthrobacter paraffineus* produces a glycolipids category of biosurfactant which can assist in the bioremediation of marine environment (Elliot et al. 2010; Shekhar et al. 2015). Further, extreme halophilic actinomycetes, *Actinopolyspora sp. DPD1* isolated from the oil production site of Sultanate of Oman can degrade hydrocarbons pentadecane, pentacosane, and eicosane at 25% of the NaCl concentrations in the marine environment. This actinomycetes can degrade short- and long-chain hydrocarbons with the further ability to degrade fluorene (Al-Mueini et al. 2007).

Dietzia maris, a marine actinomycetes isolated from the oil-polluted marine soil, efficiently degrades n-alkanes and iso-alkanes at 10% NaCl concentration (Zvyagintseva et al. 2001). It is also involved in the bioremediation of diesel at 15–17% NaCl concentrations (Borzenkov et al. 2006; Riis et al. 2003). *Dietzia maris As-13-3*, a deep sea marine actinomycetes, is involved in the bioremediation of the hydrocarbons in the presence of biosurfactant produced by itself (Wang et al. 2014). *Micrococcus luteus MN-006* degrades naphthalene in marine environment (Zhuang et al. 2003). *Micrococcus* sp. MOLA73 (UBF-P5a) and *Gordonia* sp. PETBA11, marine actinomycetes from the consortia UBF-Pycan, efficiently degrade and mineralize pyrene and PAHs in culture medium. While these actinobacteria individually cannot degrade pyrene, in consortia, the *Gordonia* sp. are more efficient in the degradation possess, using NidA3 pyrene dioxygenase enzyme. NidA3 pyrene dioxygenase is similar to the mycobacterial enzyme (Gallego et al. 2014). *Gordonia* sp. *JC11* and *JC8* of the marine origin can degrade the boat lubricants. *Gordonia* sp. *JC11* is reported as an efficient degrader of lubricant with above 50% efficiency. Among the various factors affecting degradation are emulsification and cell surface hydrophobicity of the isolate (Chanthamalee and Luepromchai 2012). *Gordonia westfalica GY40* of the marine origin produces a biosurfactant which solubilize and disperse the PAHs and oils in the environment. The common pathway for the degradation of aromatic hydrocarbon by microbes is depicted in Fig. 20.1. The combination of *Gordonia westfalica GY40* produced biosurfactant with the polyurethane foam (PUF) immobilized *Gordonia* sp. *JC11* degrades the oil and other hydrocarbons with 60–70% degradation efficiency (Laorrattanasak et al. 2016). *Micrococcus MN-006* obtained from the marine habitat is a potential actinobacteria for the degradation of PAHs and naphthalene (Zhuang et al. 2003). *Dietziasp.Q1* and *Arthrobacter* sp. *Q3* isolated from the oil-polluted saline soil are capable of degrading the diesel and other hydrocarbons in consortia. These organisms can degrade 40% of total petroleum hydrocarbon (TPH) in short time when used in consortia (Somee et al. 2018). The broth of *Gordonia sp. strain JE-1058* was spray dried to produce remediation agent (JE1058BS) supplemented with a biosurfactant for the bioremediation of the oil spills. Further, this remediating agent triggers the indigenous flora to remediate the crude oil pollution in seawater and sediments (Saeki et al. 2009). *Janibacter* sp. SB2 isolated from sea-tidal flat by the enrichment culture degraded benzene, toluene, ethyl benzene, and xylene in the presence of NH_4Cl (Jin et al. 2013). Bioremediation of various pollutants by marine actinomycetes from different habitat are depicted in Table 20.1.

20.4 Factors Influencing the Bioremediation

Marine ecosystem encompasses all extremities and hence it is difficult for any degradation process to be unanimous. The majority of the bioremediation processes are affected by the different factors illustrated in Fig. 20.2.

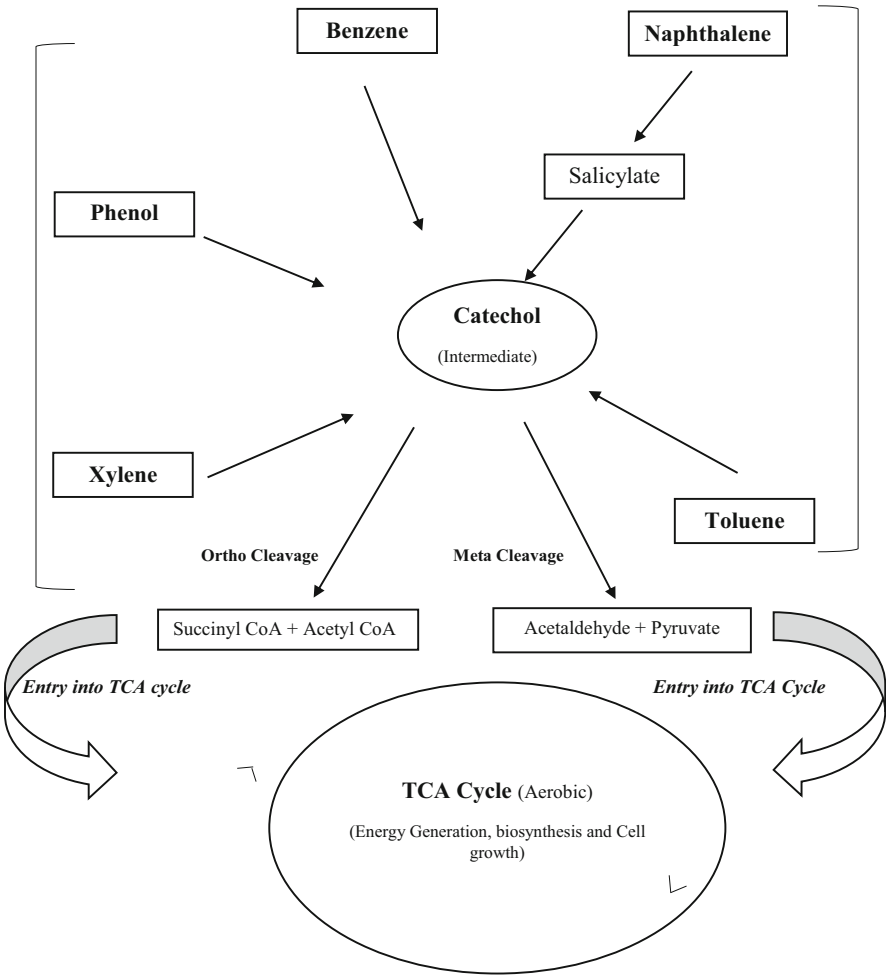


Fig. 20.1 Accessory and central aromatic hydrocarbon degradation pathways used by microbes

20.4.1 Bio-accessibility of Pollutants

For any process to be significant, the availability of pollutant molecule is quite important. Due to much dispersal and sedimentation of pollutants in seawater, the accessibility of the pollutants decreases. There are certain bioactive agents, such as biosurfactants and emulsifiers, which make pollutants available to the microbes for degradation. These additives biomolecules are produced in situ conditions during the biodegradation. Trehalose type of biosurfactant is produced by the *Rhodococcus erythropolis* and some *Nocardiopsis* spp. Sulfonylipids, fatty acids and Streptofactin type of biosurfactant are produced by the *Corynebacterium alkanolyticum*, *Corynebacterium lepus and Nocardia erythropolis*, and *Streptomyces tendae*, respectively (Mulligan 2005; Boopathy 2000).

Table 20.1 Marine actinomycetes involved in the bioremediation of various pollutants

Isolate	Habitat	Degrading compound	Reference
Rhodococcus sp. DB11	Saline oil polluted site	Octane (C_8H_{18}) degradation at 6% NaCl	Plotnikova et al. (2011)
Rhodococcus erythropolis strain DCL14	–	Octane (C_8H_{18}) degradation at 2.5% NaCl	de Carvalho and ds Fonseca (2005)
Rhodococcus sp.	–	Degradation of diesel at 10% of NaCl conc.	Borzenkov et al. (2006)
Rhodococcus erythropolis	–	Degrade Hexadecane ($C_{16}H_{34}$) at 7.5% of NaCl	de Carvalho (2012)
Rhodococcus sp.TW53	Antarctica	Lipopeptide type of biosurfactant in the marine bioremediation	Peng et al. (2008)
Rhodococcus erythropolis 3C-9	Oil polluted Mumbai coast	Glycolipid and Trehalose type of biosurfactant	Peng et al. (2007)
Rhodococcus fascians	Marine origin	remediation of marine oil spills	Sharma and Pant (2000)
Rhodococcus sp. NCIM 5126	Marine water	Bio emulsifier mediated hydrocarbon degradation at low temperature	Andrady (2011)
Rhodococcus ruber	Marine origin	Degrade the crude oil	Langdahl et al. (1996) and Kohyama et al. (2006)
Rhodococcus erythropolis BL1 and *Rhodococcus pyridinivorans* S85-2	Marine environment of Kuwait	Degrade plastic waste in marine water and sediments	Ryu et al. (2006)
Rhodococcus sp. YHLT-2		Degrade acetonitrile with help of enzyme nitrile hydratase	Sorkhoh et al. (1990)
Rhodococcus rhodochrous		Degrade short and long chain present in the petroleum. Remediate the hydrocarbons	
Nocardiopsis alba MSA10 and *Nocardiopsis lucentencis* MSA04	Marine water Oil contaminated marine sediments	Produces lipopeptide type of biosurfactant which remediate sea water	Gandhimathi et al. (2009) and Kiran et al. (2010a, b)
Nocardiopsis sp. NCIM 5124	Marine sediments	Bioremediation using degradation of hydrocarbon in crude oil	Dixit and Pant (2000a, b)
Nocardiopsis aegyptia	Marine habitat	Degrade polyesters in marine environment	Ghanem et al. (2005)
Nocardiopsis sp. 13H	Marine origin	Adsorbs the Cs^+ ions up to the 50 mM of $CsCl_2$	Sivaperumal et al. (2018)
Nocardiopsis sp. VITSISB		Adsorb radionuclei strontium (Sr^+) up to the concentration of 30 mM	Kamala et al. (2019)
		Produces biosurfactant rhamnolipid which degrade engine oil and oil spills	Roy et al. (2015)
Streptomyces sp. VITDDK1, VITDDK2 and VITDDK3	Marine origin Bay of Bengal sediments	Bioremediation of heavy metals like Cd (II) and As (V) respectively	Lakshmipathy and Kannabiran (2010); Lakshmipathy et al. (2010)
Streptomyces VITSVK9	Bay of Bengal and Tamilnadu	Remediate the heavy metals like Cr (III) and Cr (VI) by the biosorption mechanism	Saurav and Kannabiran (2011)
Streptomyces A160, *Streptomyces acrimycini* NGP, *Streptomyces*			

(continued)

Table 20.1 (continued)

Isolate	Habitat	Degrading compound	Reference
albogriseolus NGP and *Streptomyces variabilis* NGP *Streptomyces albiaxialis* *Streptomyces albidoflavus* *Streptomyces* sp. TIS6 (6H) *Micromonospora chalcea* *Micromonospora* sp. J3S17	coastal area Marine origin Sea water Marine origin Saline soil Marine origin	Bioremediation of Cu(II) and Pb (II) by the process of biosorption Degrade the diesel and other hydrocarbons at the 3–30% concentration of the NaCl Complete mineralization of nitrobenzene, phenol and toluene up to 2 M NaCl concentration Remediate the Cs^+ and Sr^+ radioactive metals Degradation of carbofuran pesticide Remediate the Cs^+ and Sr^+ radioactive metals	Yadav et al. (2009); Selvam and Vishnupriya (2013a, b) Kuznetsov et al. (1992) Ai et al. (2008) Sivaperumal et al. (2018) and Kamala et al. (2019) Jayabarath et al. (2010) Sivaperumal et al. (2018); Kamala et al. (2019)
Arthrobacter sp. SN17 *Arthrobacter paraffineus*	Marine habitat –	Octane (C_8H_{18}), Naphthalene, biphenyl, salicylate, o-Phthalate, Gentisate, and Phenanthrene at the 3–6% of NaCl concentration Produces a glycolipids type of biosurfactant helps in bioremediation	Plotnikova et al. (2011) Elliot et al. (2010); Shekhar et al. (2015)
Actinopolyspora sp. DPD1	Sultanate of Oman	Degrade the hydrocarbons like pentadecane, pentacosane and eicosane at 25% of the NaCl concentration	Al-Mueini et al. (2007)
Dietzia maris *Dietzia* spp. *Dietzia maris*As-13-3	Oil polluted marine soil Deep sea marine habitat –	Degradation of n-alkanes and iso-alkanes in the presence of 10% NaCl concentration Bioremediation of diesel pollution at 15–17% NaCl concentration Bioremediation of the hydrocarbons in the presence of biosurfactant	Zvyagintseva et al. (2001) Borzenkov et al. (2006); Riis et al. (2003) Wang et al. (2014)
Micrococcus luteus MN-006 *Micrococcus* sp. MOLA73 *(UBF-P5a)* and *Gordonia* sp. PETBA11	Marine environment Marine habitat	Degrade the naphthalene Degrade and mineralize the pyrene and PAHs	Zhuang et al. (2003) Gallego et al. (2014)

(continued)

Table 20.1 (continued)

Isolate	Habitat	Degrading compound	Reference
Gordonia Sp. JC11 and JC8 *Gordoni awestfalica GY40*	Marine origin	Degrade the boat lubricants Biosurfactant solubilize and disperse the PAHs and Oils	Chanthamalee and Luepromchai (2012) Laorrattanasak et al. (2016)
*Janibacter*sp. SB2	Sea-tidal flat	Degrade the BTEx (Benzene, Toluene, Ethyl benzene and xylene)	Jin et al. (2013)

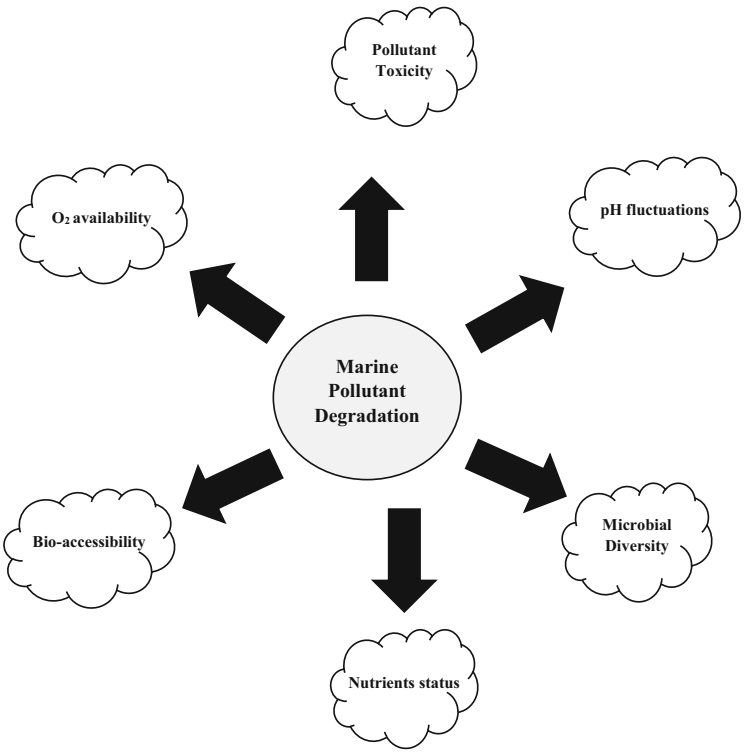


Fig. 20.2 Factors influencing the pollutant biodegradation in marine environment

20.4.2 Extent of Aerobic Conditions

Majority of marine actinomycetes are aerobic and thus need ample amount of oxygen. In aerobic degradation, about 90% of the degradation efficiency is achieved, which may reduce to only 25% under anaerobic conditions. *Rhodococcus* and *Mycobacterium* aerobically degrade hydrocarbons (Nicolau et al. 2009; Song et al.

2011). Marine actinomycetes have not been frequently reported for anaerobic degradation. Unfavorable conditions are likely to be generated in the excess of pollutants. The key enzymes involved in the aerobic biodegradation are oxygenases and peroxygenases. These enzymes initially attack on the pollutant converting it into the precursor metabolites for other pathways. Moreover, P450 alkane hydroxylases associated with the family of monooxygenases can degrade majority of PAHs and chlorinated compounds in the seawater (Das and Chandran 2011).

20.4.3 Toxicity of the Pollutants

Some pollutants are toxic to the microbes and thus can be limiting factor for the degradation. In some cases, the compounds itself are not toxic to the microorganisms but the intermediates produced during the degradation are toxic to the bacterial strains. However, such compounds may be precursor for other microorganisms in the process.

20.4.4 pH of the Surrounding

The degradation of the pollutants may change the pH of the surrounding in an unpredictable manner and thus can limit the degradation process. Actinomycetes require optimum pH for their activity, but unfavorable conditions generated due to pH fluctuations can limit the bioremediation.

20.4.5 Microbial Distinctness

Microbial diversity in the polluted habitat is significant as different organisms possess a different set of pathways and remediation conditions. Moreover, a synergistic approach can be adopted for the degradation and proliferation of the microorganisms. Microbial distinctness allows complete degradation of pollutant if a particular species is able to degrade at a moderate level (Sabra et al. 2010; Gkorezis et al. 2016). Some other factors like temperature, concentration of the nutrients, in situ and ex situ conditions, effect of inhibitors, adhesion properties of cells, catabolic repression, ecological behavior, and external environmental factors limit or enhance the biodegradation process in marine ecosystem.

20.5 Methods for the Evaluating the Bioremediation

Detailed information about the methods used for the assessment of bioremediation process is given in Table 20.2.

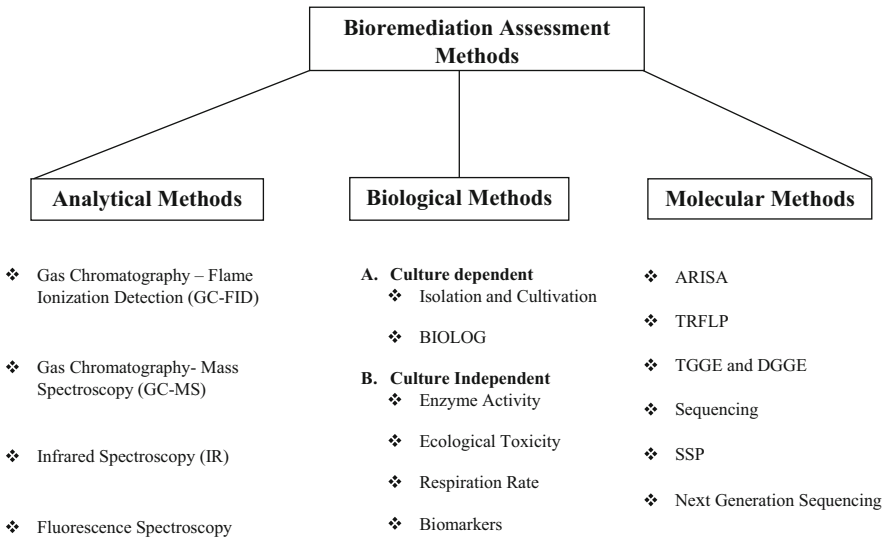
Table 20.2 Methods for the evaluation of bioremediation

Sr. no.	Method	Compounds	Limitation	References
1.	Physicochemical methods			
	A. Gas chromatography-flame ionization detection (GC-FID) B. Gas chromatography – mass spectrometry (GC-MS) C. Infrared spectroscopy D. Fluorescence spectroscopy	Hydrocarbons and petroleum oil degradation Different environmental samples and PAH TPH estimation PAH and other pollutant estimation using fluorescence	High operational time, difficulty in calibration and cost Very less solvents are available for the sample extraction, time and cost consuming Insensitive to some of the aromatic hydrocarbons Complex compounds cannot be estimated	Sherma and Zweig (1972); Wang and Fingas (1995); Saari et al. (2010) Peterson et al. (2002); Chuang et al. (2003); Poster et al. (2006); Thornton et al. (2011) Aske et al. (2001); Lambert et al. (2001); Forrester et al. (2013); Webster et al. (2016) Aldstadt et al. (2002); Greason (2009); Barnes (2009)
2.	Biological methods			
	A. Culture dependent a. Cultivation and isolation of actinomycetes b. Biolog plates B. Culture independent a. Enzyme activity b. Soil respiration test c. Ecotoxicity tests C. Molecular techniques a. Terminal restriction fragment length polymorphism (TRFLP) b. Automated ribosomal intragenic spacer analysis (ARISA) c. Single-strand conformation Polymorphism	Initial and after pollutant degradation microbial count Metabolic activity of degraders microorganisms Biodegradation of PAHs and estimate the enzyme (dehydrogenase) activity Measurement of produced or consumed gases after PAH and other pollutant degradation Toxicity of the pollutant is checked after degradation Community analysis of polluted environmental samples Community	Non-culturable microbes cannot be cultured Insufficient cell density result into false result Presence of elemental compound affect the enzyme activity Respiration may not corresponds to the degrader organisms Ecotoxicity may not be related to the degrader organisms Time consuming and laborious Limited genetic information is obtained Less accuracy corresponds to the	Schlegel and Zaborosch (1993); Balba et al. (1998) Hill et al. (2000); Widmer et al. (2001) Baran et al. (2004); Gianfreda et al. (2005) Hinchee and Ong (1992); Torstensson (1996); Kim et al. (2005); Dawson et al. (2007) Lanno et al. (2004); Mooney et al. (2013); Shahsavari et al. (2017) Rastogi and Sani (2011) Kovacs et al. (2010); Gobet et al. (2014) Schwieger and

(continued)

Table 20.2 (continued)

Sr. no.	Method	Compounds	Limitation	References
	(SSCP) d. Denaturant gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) e. Sanger sequencing f. Next-generation sequencing	richness of polluted environmental samples Community complexes of polluted environmental samples Community structure changes assessment of environmental samples 16S rRNA based phylogenetic identification of microorganisms from polluted environmental samples Microbial profile from the direct environmental samples	instrument used Limited size of fragments cannot correspond to the phylogenetic analysis Inadequate method for heterogeneous sample analysis Cultivability is absent as only environmental DNA is considered	Tebbe (1998); Malik et al. (2008) Muyzer (1999); Malik et al. (2008) Lakshmi (2010); Shokralla et al. (2012) Khudur et al. (2018)



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