Environmental Chemistry for a Sustainable World 50

K. M. Gothandam
Shivendu Ranjan
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Preface

Think Circular

This book is the third volume on Environmental Biotechnology, published in the series Environmental Chemistry for a Sustainable World. Environmental quality is crucial for the health and survival of humans and other living organisms. In particular, in the context of increasing globalization, urbanization and industrialization, it will soon be mandatory to waste nothing and, as a consequence, to develop a circular economy in which all elements are recycled. For that, biotechnological techniques such as biodegradation, bioremediation, biomonitoring, biotreatment, biofiltration, phytoremediation and immobilization can be used to convert waste into 'golden' products and chemicals. Environmental biotechnologists thus identify, develop, improve and engineer a suitable technology for remediation and other applications. Chapters review the biodegradation of plastic waste, bioremediation of S-triazine herbicide, the effect of pesticide on birds, microbial biosensor for the detection of pollutants, production of polyhydroxyalkanoates from industrial wastewater treatment, concerns in membrane distillation process, applications of immobilized microalgae, applications of marine microbial biosurfactants and biochar for plant production (Fig. 1).

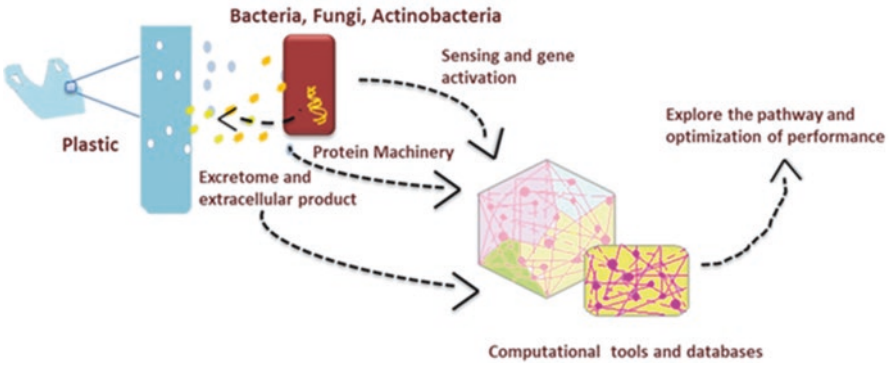

Fig. 1 Microbial action on plastic and degradation pathway prediction by using various tools (Alka Kumari et al., Chapter 1)

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Chapter 1
Microbial Degradation of Plastics and Its Biotechnological Advancement

Alka Kumari, Doongar R. Chaudhary, and Bhavanath Jha

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Abstract Plastic waste is building up in the environment with an alarming rate. Conventionally, plastic waste is dumped in landfills, incinerated, or recycled; these methods release another form of toxic chemicals and plasticizers like furans, bisphenol A, phthalate, and vinyl chloride in the environment. Nature itself recuperates and maintains sustainable conditions to some extent although it is extremely slow. Exploring the microbes from the environment and enhancing their plastic degradation capability through advanced biotechnological tools would provide an eco-friendly solution. This chapter focused on recent reports on plastic biodegradation emphases on preceding dogmas of plastic hazard and the understanding of standard evaluation methods along with their biogeochemistry in the environment by the microorganisms.

Keywords Biodegradation · Biogeochemical process · Enzymes · Genomics · Microbial degradation · Metabolomics · Plastic

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1.1 Introduction

Plastic discovered as a material element has revolutionized many sectors due to their application versatility (Singh et al. 2011). These synthetic polymers from the petroleum fraction have replaced many natural materials used before their discovery; however, their recalcitrant nature resulted in long persistence in the environment that ended up in landfilling and burning; as well seepage in different ecosystems has now become a global concern (Thompson et al. 2009a; Goudie 2018). These wastes act as key indicators of human activity (Rochman 2015; Zalasiewicz et al. 2016). Industrial development and increased population are the foremost contributors to plastic waste generation (Singh et al. 2011). The traditional plastic waste management system includes landfilling, burning, and recycling, resulting in infertile land and toxic chemical evolution in the environment. Only around 15–25% of the plastic waste is recycled in Europe and recycling below 5% in the USA, although the remaining persist as such in the environment (Barnes et al. 2009). Globally, 8% of the petroleum fraction is used to produce plastic, where 4% used as raw material and 4% in the energy required for production (Hopewell et al. 2009; Thompson et al. 2009a). The major proportion of plastics is used in packaging, which is often discarded rapidly, which creates an unsustainable situation and demands control measures.

To decrease the plastic discards in the environment, the alternative approaches are to produce biodegradable plastics and to isolate microbes from the environment that are capable to degrade plastics (Wierckx et al. 2015; Kumari et al. 2019; Kawai et al. 2019). Many biodegradable and bio-based polymers have been developed, but complete replacement of plastic is impossible due to their high production cost and application limitations. Besides this, microbial degradation of the synthetic polymer will be innovative to the world; however, the efficiency of this eco-friendly measure for reducing plastic waste is questionable (Tolinski 2011). In nature, the process of biodegradation is continuously occurring to maintain planet sustainability. However, the rate of waste production is much higher than that of natural degradation processes.

Bioremediation technology will provide a sustainable solution for rising plastic waste in the environment. The research on bioremediation studies is developing, and many enzymes from microbes are capable of destabilizing and degrading recalcitrant synthetic plastics (Bhardwaj et al. 2013; Wei and Zimmermann 2017; Kumari et al. 2017). Microbial enzymes are developing as biocatalytic agents for the remediation and recycling processes of plastics, by which valued raw materials can be recovered and produced (Araújo et al. 2007). Microbial plastic degradation will be an eco-friendly approach for reducing plastic waste as well as a solution to the distressing situation due to plastic waste generation (Devi et al. 2016).

To date, no feasible biodegradation processes have been developed that can be used on a large scale. Research advancement in the genomics and metabolomics area concords to cognize the microbial ability to degrade polymeric wastes and predict degradation pathways (Dvořák et al. 2017). Here, recent findings and possible plastic waste remediation strategies have been summarized, and future

integrative research directions have been highlighted. In addition, for developing plastic waste remediation technology, improved waste collection and segregation methods are central.

1.2 Plastic Properties and Its Types

The term plastic is coined from the French word *plastique* which itself means the ability to mold (Albus et al. 2006). A polymeric plastic is made from phenol and formaldehyde, Bakelite discovered by Leo Baekeland in the year 1909, which was the first completely synthetic material with numerous applications. Polyethylene was accidentally discovered in the year 1898, not taken in much notice was rediscovered in 1933 by another accidental mixing of ethylene and benzaldehyde at high temperature and pressure (Baker 2018). Now, plastics entered in the daily human life, from the very initial use of cellulite material in place of today's plastic. The discovery and rediscovery of plastic has been continued with varying raw material and conditions to produce more and more versatile material. This development of material elements has been used for many applications from packing, construction, and medical advancement (Andrady and Neal 2009; Saeedi et al. 2011). Basically, plastics are polymer of high molecular weight, and stable carbon backbones make them hydrophobic and nondegradable in nature. However, after polyethylene discovery, many polyolefins were developed and introduced in the market by varying the arrangement of atoms and modifying or changing monomeric units in the chain and various forms of plastic discovered.

Plastics have been classified on the basis of origin, molding ability, recyclability, and monomers (Van Krevelen and Te Nijenhuis 2009; Nicholson 2017) (Fig. 1.1). Plastics are classified as natural and synthetic on the basis of their origin, where it is understandable that natural plastics are made up of natural raw materials (i.e., cellulose, lignin, silk, and protein) and synthetic ones are man-made besides from an unnatural source (i.e., polyethylene, polyester, polystyrene, and polyvinyl chloride) (Jabbar 2017). Another classification system is thermal or molding properties where one is thermosetting (cannot be molded by heating) (i.e., melamine, phenol formaldehyde and polyurethane) and another is thermoplastic (molded after heating) (i.e., polyolefins, vinyl polymers and polycarbonates) that also defines molding ability and recyclability. They are also classified based on the monomers: homopolymer (chain of same monomers) (i.e., polyethylene, polypropylene, polystyrene, and polyvinyl chloride) and copolymer (chains of two or more types of monomers) (i.e., polyethylene terephthalate, nylon, and polyurethane). The synthetic thermoplastics were further classified based on their recyclability by coding them with numerical values inside a closed triangular arrow symbolizing their recyclability (Society of the Plastics Industry coding) [poly ethylene terephthalate (1), polyvinyl chloride (3), high-density polyethylene (2), low density polyethylene (4), polypropylene (5), polystyrene (6), polycarbonate/polyurethane (7)] (Halden 2010; Garcia and Robertson 2017; Rahimi and García 2017) (Fig. 1.2).

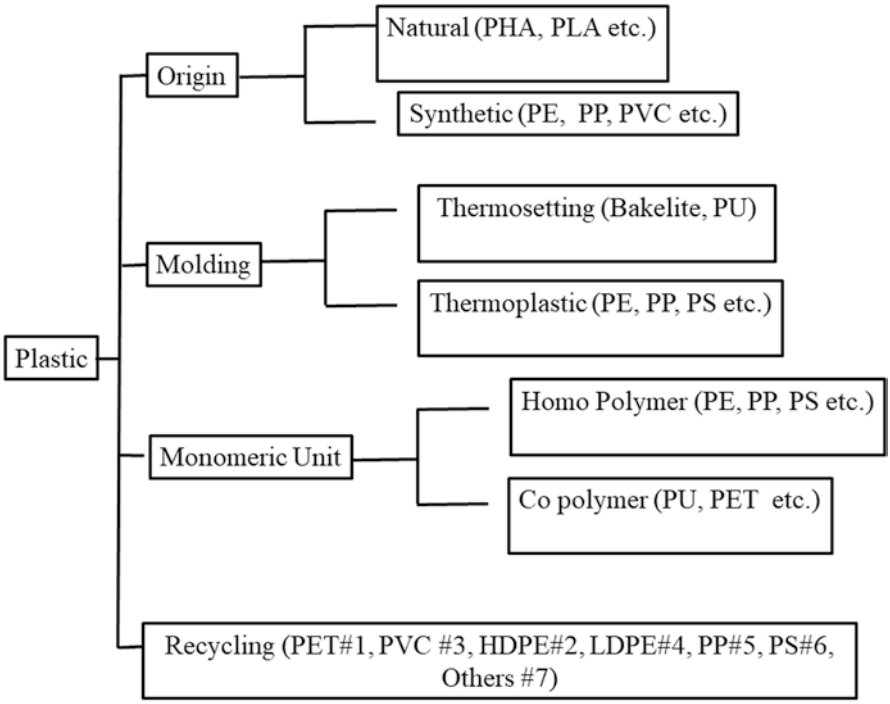

Fig. 1.1 Classification of plastics (*PHA* polyhydroxyalkanoate, *PLA* polyhydroxy lactonoate, *PE* polyethylene, *PP* polypropylene, *PVC* polyvinylchloride, *PS* polystyrene, *PU* polyurethane, *PET* polyethylene terephthalate, *HDPE* high-density polyethylene, *LDPE* Low-density polyethylene)

The residual fragmentation and chemical groups of plastics vary considerably when exposed to a natural environment depending on environmental factors, form of polymer, polymerization technique, and additives used. These low molecular weight components of polymers may leach easily from plastic products in the environment and cause unconfined risks (Hahladakis et al. 2018). Assessment of exposure risk is required in the context of plastic waste pollution, degraded products, additives, and their toxicity. From the CLP (classification, labeling, and packaging) regulation by the European Union Council (2008), polymers have been classified based on their hazardous effects. They classified hazard level of polymers based on the risk from their monomers such as either carcinogenic, mutagenic, or both (category 1A or 1B) (i.e., polyurethanes, polyacrylonitriles, polyvinyl chloride, epoxy resin, and styrene copolymers) (Aulmann and Pechacek 2014). Since, the residual monomer amounts vary among different products and polymers and are accordingly hazardous (Halden 2010; Lithner et al. 2011).

Polyolefin is manufactured through polymerization reaction of monomers (i.e., polyethylene and polypropylene) or condensation reaction of carboxylic acid and an alcohol or amine group between their monomer (i.e., polyester or polyamide). The polymerization reaction is a highly exothermic process, where the monomers or

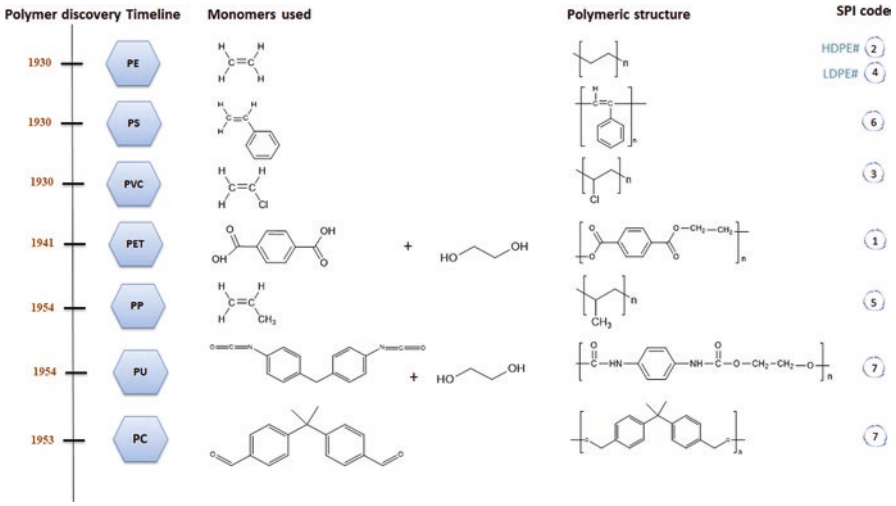

Fig. 1.2 Plastic discovery timeline and characterization based in their monomers and SPI (Society of the Plastics Industry) coding (*PE* polyethylene, *HDPE* high-density polyethylene, *LDPE* low-density polyethylene, *PVC* polyvinylchloride, *PET* polyethylene terephthalate, *PS* polystyrene, *PP* polypropylene, *PU* polyurethane, *PC* polycarbonate)

starting material should be of high purity in the presence of a metal oxide or chloride catalyst under high pressure and temperature (Goodship 2007). Polyethylene is classified based on its density and branching pattern (Andrady and Neal 2009). Polyethylene and polyethylene terephthalate are the most common forms of plastics, primarily used for packaging water bottles and short consumption cycle products, made up of ethylene monomers chains. Degradation of these polymers is more challenging than others because of their easy dispersal in the environment due to their large application. Polyethylene terephthalate is commercially produced by reacting ethylene glycol with terephthalic acid to produce bis-hydroxyethyl terephthalate followed by the polymerization of bis-hydroxyethyl terephthalate under high temperature and pressure (Park and Kim 2014).

Polyurethane lays between thermosetting polymers, and thermoplastic is a highly durable polymer composed of urethane units joined by carbamate links. The urethane units were synthesized by reacting polyol with diisocyanate. The range of polyurethane can be produced by using different polyol with diisocyanate in the presence of suitable catalysts and additives (Szycher 1999). Polyurethane is a rigid or flexible polymer resulting from the substitution and monomer arrangement pattern. Polyurethane has SPI (Society of the Plastics Industry) code 7 showing a non-recyclable nature (Engels et al. 2013; Rahimi and García 2017).

Polyvinyl chloride, the third largest plastic product, needs to get extra attention because of its carcinogenic monomer and often constituted of hazardous additives. Polyvinyl chloride is also known as “poison plastic” because of the toxic additives and vinyl chloride leaching throughout its entire lifecycle. Also, polyvinyl chloride

has SPI (Society of the Plastics Industry) code 3, indicating its low recyclability competence. Polyvinyl chloride is not used for various applications like storing or packaging food materials or for medical purposes (Halden 2010).

1.3 Plastic Waste Threats to Well-being

Within decades of plastic discovery, it seems to be present everywhere from the coastal to the terrestrial environment. Globally, plastic production has expanded up to 300 million metric tons, and at this rate, it is expected to reach approximately 33 billion tons by 2050 (Sussarellu et al. 2016), where polyethylene alone produced around 140 million tons per year, contributing major part of plastic waste (Rochman et al. 2013) also exemplify 35 kg of annual plastic production is equal to the 7 billion human biomass on earth (Zettler et al. 2013). This unwanted rise of plastic waste in the environment is recognized as "white pollution" (Liu et al. 2014). The traditional practices of waste management systems also fail due to a lack of seriousness and improper segregation structure for plastic waste collection. Annually, approximately 12 million tons of plastic waste enter the ocean (Jambeck et al. 2015; Li et al. 2016). The International Pacific Research Centre modeled plastic waste accumulation across the Hawaii island and termed accumulated plastic patches near the North Pacific Ocean as "Great Pacific Garbage Patch" and "the Trash Vortex" in the southern hemisphere, evidenced by the careless use and dumping of plastic waste in the ocean (Eriksen et al. 2014). Plastics impend and expanse out up to the space, as they have now been carried across the solar system by spacecraft and satellites. Van Franeker and Bell (1988) reported that plastic particles are common pollutants in stomachs of Wilson's storm petrels (*Oceanites oceanicus*) and Cape petrels (*Daption capense*) on the Antarctic continent and being the reason for their death before fledging. Land also a potential sink for plastic wastes affecting land fertility, groundwater movement and local habitat however, very least discussed. The marine environment is exposed and has been widely studied in the context of plastic pollution. Plastic is observed everywhere, from floating on open sea to deep surface, sediments, and inside lives of sea (Thompson et al. 2009b). The large quantity of plastic comes from direct dumping, seeping from landfill sites, sewage, or accidents.

Plastics in the environment weathered due to natural or anthropogenic events into smaller sizes (<5 mm) called microplastics (Barnes et al. 2009). However, the extent of pollution by microplastics is much more widespread than earlier assumptions. A total of around 35,500 metric tons of microplastic floats in the ocean have been estimated globally (Eriksen et al. 2014), while Wieczorek et al. (2019) reported the presence of microplastics in the 46% of fecal pellets of salp in an experiment where microplastic supplied to environmentally relevant concentrations. These micro-sized plastics are ingested by organisms like amphipods, lugworms, barnacles, sea cucumbers, and mussels causing their digestive system to bleed (Avio et al. 2017; Barboza et al. 2018). In a laboratory study, it was reported that marine fishes

assimilate chemicals such as polyaromatic hydrocarbons, polychlorinated biphenyls, and polybrominated diphenyl ethers and suffer liver toxicity (Rochman et al. 2013). Plastic waste in the ocean also acts as a route for transporting many sessile invasive species and spreading them to different ecosystems.

On the other hand, plastic products are mixed with additives and plasticizer to get desirable properties such as softening, polishing, heat, and UV stabilizers that add more to recalcitrant and toxicity. Bisphenol A and Di-(2-ethylhexyl)-phthalate are common additives used in making plastic products where Bisphenol A is a unit of polycarbonate plastics, where they slowly leach out over time or exposed to heat or liquid. Numerous combinations of plastics and additives are available that mark plastic pollutants prone to anonymous risk levels (Thompson et al. 2009a). The chemical mixture and toxicity will vary based on the duration and environmental exposure. These additives and plasticizers risk life to blindness, cancer, endocrine disruption, effective reproductive system, and embryo developmental stages (Table 1.1) as well, and disintegration into microplastics causes ingestion and accumulation in the tissues that pass through the food chain (Webb et al. 2012; Lusher et al. 2015). Plastic-derived polybrominated diphenyl ethers are found in the abdominal adipose tissue of seabird (Tanaka et al. 2013). Boerger et al. (2010) reported plastic pieces in 36% of planktovorous fish with an average of 2.1 plastic pieces found per fish from the North Pacific Central Gyre.

Rochman et al. (2015) reported that the plastic debris was found in 28% and 55% of Indonesian fish and shellfish and in the USA 67% in fish and 33% in shellfish sampled. The plastic consumed by aquatic organisms indirectly comes to our plate. The subsequent plastic application in the era of modernization plastic and its additive residues definitely present in our body via direct exposure or subsequent food webs (Andrady 2011).

1.4 Biodegradation and Natural Cycling of Plastic Waste

Biodegradation is a process of transformation or alteration of the chemical structures through the metabolic or enzymatic action of the living organism present in the environment (United States Environmental Protection Agency 2009). The process of biodegradation and re-mineralization of waste continuously occurs in nature to hold onto the planet sustainability, but the rate of waste generation is much higher than the natural cycle that takes hundreds to thousands of years to complete, therefore considered as recalcitrant material (Lardjane et al. 2013). Research interest in biodegradation studies was started in 1982, involving classical research and facing challenges due to technological limitations (Boopathy 2000). Studies indicate that microorganisms act differently over different plastics (Nauendorf et al. 2016). The indigenous microbial community and local environmental conditions play a major role in the biodegradation rate, which determines the biogeochemical events (Sarkar et al. 2016). Microorganisms play a major role in mediating elemental biogeochemical cycles by mineralizing the organic material into inorganic through their

Table 1.1 Common additives used in various plastic and their hazards

S. No.	Additive	Function	Plastic	Risk	References
1.	Vinyl chloride	Paint, monomer for Polyvinylchloride	Polyvinylchloride, furniture, wall covering	Hepatic angiosarcomas, mutagenic	Criscuolo et al. (2013) and Lotti (2017)
2.	Butylated hydroxytoluene	Prevent oxidation	Polyolefins	Teratogen, mutagenic, carcinogenic, growth retardation and dermatitis	Nieva-Echevarría et al. (2015)
3.	Phthalates	Soften plastic in food packaging	Polyvinylchloride	Endocrine disruption, asthma, developmental and reproductive defects	Meeker et al. (2009)
4.	Bisphenol A	Adhesives, electronic coatings	Polycarbonate	Carcinogen, affect immune system, early onset of puberty and endocrine disruption	Erler and Novak (2010)
5.	Polybrominated diphenyl ethers	Flame retardant,	Polyurethane, Polyolefins	Carcinogen, fetal malformations, hypatotoxicity, thyroid defect	Bjermo et al. (2017)
6.	Nonylphenol	Stabilizer in plastic, food packaging	Polyolefins	Breast cancer and endocrine disruptor	Sifakis et al. (2017)
7.	Polychlorinated biphenyls	Non flammability, chemical stability, electrical insulation	Polyolefins, furniture, wall covering	Endocrine disrupters and carcinogen	Akovali (2012)

metabolism (Keswani et al. 2016). Their simple structure and fast division rate make them capable of adapting to the environment (Zeglin 2015). Microorganisms are ubiquitous in nature and can adapt to the external environment and nutrient availability and communicate through signals like acyl homoserine lactones to other cells in the region (McCarty and Ledesma Amaro 2018).

Various microorganisms have been reported, such as protist, fungi, yeast, and bacteria for the biodegradation capability for different types of plastics (Table 1.2). The ocean has a wide range of environmental factors, such as temperature, salinity, pH, hydrodynamic, and nutrient limiting conditions that led to the evolution of various marine habitats and species diversity (Takai and Nakamura 2010). Marine bacteria have always been of research interest because of their high biodiversity and

Table 1.2 Microbial degradation of plastics and tools used in the study

S. No.	Polymer	Microorganisms	Experimental period	Enzyme group	Product of degradation	Analytical evidence	References
1.	Preheated Starch-Polyethylene	*Streptomyces badius, S. setonii, S. viridosporus*	3 weeks	Extracellular culture fluid	Carbonyl group formation	FTIR, GPC, tensile strength	Pometto et al. (1992)
2.	Low density polyethylene, PS, Polyvinylchloride and urea-formaldehyde resin	*Aspergillus niger, Fusarium sp., Penicillum sp., Candida sp., Clostridium sp., Bacillus cereus, Bacillus subtilis*	32 years	Hydrolase	Stretching vibration of C=C bond and carbonyl group formation	SEM, FT-IR, Differential Scanning Calorimetry, X-ray diffraction	Otake et al. (1995)
3.	Nylon 6	*Aspergillus terreus*	8 days	Lipase	Peroxidase-oxidase reaction	SEM, FTIR, tensile strength	Kiumarsi and Parvinzadeh (2010)
4.	Polyethylene and nylon-66	–	2–3 days	Laccase-mediator system	–	HT-GPC,FTIR, Elongation, tensile strength	Fujisawa et al. (2001)
5.	Urethane compounds	*Rhodococcus equistrain*	36 hours	Urethane hydrolase (amidase/esterase)	Toluene-2,4-dicarbamic acid dibutyl ester, Methylene bisphenyl dicarbamic acid dibutyl ester and Hexamethylene dicarbamic acid dibutyl ester	HPLC, GC-MS	Akutsu-Shigeno et al. (2006)

(continued)

Table 1.2 (continued)

S. No.	Polymer	Microorganisms	Experimental period	Enzyme group	Product of degradation	Analytical evidence	References
6.	Nylon-6	*Bjerkandera adusta*	60 days	Manganese peroxidase, lignin peroxidase	6-amino hexanoic acid, Ɛ-caprolactam	Molecular weight determination, viscosity and differential scanning calorimetry, Elemental analysis, HPLC	Friedrich et al. (2007)
7.	Poly ethylene terephthalate and polyamide 6,6	*Fusarium solani pisi*	24–48 hours	Cutinase	Terephthalic acid; amines	Site directed mutagenesis of active site	Araújo et al. (2007)
8.	Poly ethylene terephthalate	*Humilica insolens, Pseudomonas mendocina and Fusarium solani*	96 hours	Cutinase	Terephthalic acid and ethylene glycol	Weight loss, crystallinity, HPLC, DSC, SEM, Kinetic Studies	Ronkvist et al. (2009)
9.	Polyethylene	Marine environment	40 weeks	–	–	Surface area, tensile strength	O'Brine and Thompson (2010)
10.	Nylon-6	*Agromyces* sp.	1 and 4 hours	Nylon hydrolase	6 Aminohexanoic acid	Gas cluster secondary ion mass spectrometry, X-ray crystallography, Circular dichroism spectra	Negoro et al. (2012)
11.	Polyethylene	*Rhodococcus ruber*	30 days	Laccase	–	FTIR- ATR, GPC, TGA, Quantitative PCR	Santo et al. (2013)

12.	Low density polyethylene	*Kocuria palustris*, *Bacillus pumilus*, *Bacillus subtilis*	30 days	–	–	Reduction assay, viability test, cell hydrophobicity, SEM, FTIR, Protein content,	Harshvardhan and Jha (2013)
13.	Polyethylene	Microbial community (Plastisphere)	–	–	–	SEM, next-generation sequencing	Zettler et al. (2013)
14.	Thermostable polyethylene terephthalate	*Thermobifida fusca*	–	Cutinase	–	Circular dichroism spectra	Roth et al. (2014)
15.	Poly ethylene terephthalate	*Saccharomonospora viridis*	3 days	Thermostabilized polyesterase	Terephthalic acid	SEM, site-directed mutagensis, circular dichroism, LC-IT-TOF-MS, HPLC	Kawai et al. (2014)
16.	Poly ethylene terephthalate	*Saccharomonospora viridis*	–	Cutinase	–	Differential scanning fluorimetry	Miyakawa et al. (2015)
17.	Poly ethylene terephthalate, Poly caprolactone	*Fusarium oxysporum*	–	Cutinase	–	Gene cloning and expression, X-ray crystallography	Dimarogona et al. (2015)
18.	Poly ethylene terephthalate	*Fusarium oxysporum*	–	Cutinase	Terephthalic acid, mono(2-hydroxyethyl) terephthalate	FT-IR, ATR, XPS, DSC, TGA, HPLC, Tensile tests, SEM,	Kanelli et al. (2015)

(continued)

Table 1.2 (continued)

S. No.	Polymer	Microorganisms	Experimental period	Enzyme group	Product of degradation	Analytical evidence	References
19.	Poly(butylene adipate-co-butylene terephthalate	*Thermobifida cellulosilytica*	5 hours	Cutinase	Adipic acid	Gene cloning and expression, HPLC	Perz et al. (2015)
20.	Synthetic polyesters	*Clostridium botulinum*	4 months	Esterase	Benzoic acid, terephthalic acid, mono(2-hydroxyethyl) terephthalate, and hydroxyethylbenzoate	NMR, HPLC/MS, circular dichroism spectroscopy, X-ray diffraction, cloning, and expression	Perz et al. (2016a)
21.	Poly ethylene terephthalate, Poly(butylene adipate-co-butylene terephthalate	*Clostridium hathewayi*	35 days	Cutinases	Bis(2-hydroxyethyl) terephthalate, terephthalic acid	Gene cloning and expression, circular dichroism, HPLC	Perz et al. (2016b)
22.	Poly(butylene adipate-co-butylene terephthalate	*Clostridium hathewayi*	14 days	Esterase	Bis(2-hydroxyethyl) terephthalate, mono(2-hydroxyethyl) terephthalate, terephthalic acid, 2-hydroxyethyl benzoate, benzoic acid	HPLC, gene cloning and expression, crystallization, and modeling	Perz et al. (2016c)
23..	Plastic marine debris	Sediments	100 days	Oxic and anoxic marine slurry of sediment and water	–	Epifluorescent microscope, cell density, SEM, Mass loss, Raman, contact angle	Nauendorf et al. (2016)

24.	Polyethylene terephthalate	*Ideonella sakaiensis*	15 days	Polyethylene terephthalate, mono-2-hydroxyethyl terephthalate	Terephthalic acid, Bis(2-hydroxyethyl) terephthalate, Mono(2-hydroxyethyl) terephthalate	Carbon dioxide evolution, weight loss, SEM, NMR, HPLC, transcriptomic expression	Yoshida et al. (2016)
25.	Polyethylene, polystyrene	*Rhodococcus ruber*	3 days	Laccase, extracellular oxidases, and putative dehydrogenases	–	FTIR,GPC, transcriptomics and lipidomics expression	Gravouil et al. (2017)
26.	Polycaprolactone and Polyvinylchloride films	*Aspergillus brasiliensis*, *Penicillium funiculosum*, *Chaetomium globosum*, *Trichoderma virens*, *Paecilomyces variotii*	28 days	–	–	Morphological observation, mass loss, optical microscopy, and SEM	Vivi et al. (2018)

ATR-FTIR Attenuated total reflectance-Fourier transform infrared, *GPC* gel permeation chromatography, *XRD* X-ray diffraction, *SEM* scanning electron microscopy, *NMR* nuclear magnetic resonance, *LC IT TOF MS* liquid chromatograph ion trap time of flight mass spectrometer, *HPLC* high performance liquid chromatography, *DSC* differential scanning calorimetry, *HT-GPC* high temperature gel permeation chromatography

unique adaptation to the sea environment. Moreover, they are considered to function as pioneering surface colonizers that contribute to the initiation of biogeochemical cycling and biodegradation processes and have still many unseen potential to be discovered (Dang and Lovell 2016). Nutrient scarce conditions in the ocean allow microbes to adapt easily and utilize whatever nutrients present in their surroundings might surge their capability to adhere and deteriorate materials. On the other hand, the addition of starch or other supplements provides contamination opportunities that would hinder the degradation process (Satpute et al. 2010). There have been various microorganisms involved in the deterioration of different types of synthetic polymers that have been reported to deteriorate and utilize different plastics (Table 1.2). In vitro, marine bacterial strains *Rhodococcus ruber* reported to degrade 8% of the dry weight of plastic after 30 days of incubation (Orr et al. 2004). Extracellular enzymes from *Streptomyces* species were found to cause structural changes in preheated starch-polyethylene in 3 weeks, and different strains showed different levels of polyethylene degradation (Pometto et al. 1992). These would be the footsteps of bioremediation research where the characterization of enzymes extricating different plastic structures would unveil their mechanism of action. Also, initial stresses such as heating or photo-oxidation to plastic films induce the brittlement and release of low molecular weight and carbonyl groups in the material which further help in the microbial degradation process (Eyheraguibel et al. 2017). The gut microbiome of mealworms was found to be capable of degrading polyethylene and a mixture of polyethylene and polystyrene, where two bacterial species, *Citrobacter* sp. and *Kosakonia* sp., are associated with the degradation of polyethylene and polystyrene in the gut of mealworms (Brandon et al. 2018). The low molecular weight oxidation products from polyethylene can be utilized by microorganisms, but the exact molecular weight and fragment size of released products are not known (Koutny et al. 2006).

The process of microbial degradation of plastic can take place either aerobically or anaerobically based on the nature of microorganisms executing the process. In general, it is known that the degradation product in the aerobic process is carbon dioxide and water; however, in anaerobic processes, methane will be produced (Eubeler et al. 2009). The basis of mechanism behind the structural destabilization and degradation begins with the action of extracellular oxidoreductive enzymes (lignin peroxidase and manganese peroxidase); however, detailed characterization of these enzymes is still lacking (Kumari et al. 2017). The structural and functional group changes, appearance or disappearance of bonds, reactivity, and reduction in the hydrophobicity of the plastic surface are the main aspects of the initiation of biodegradability, where variation in other factors such as molecular weight, porosity, tensile strength, and crystallinity suggests microbial action (O'Brine and Thompson 2010; Santo et al. 2013; Harshvardhan and Jha 2013; Kanelli et al. 2015). In a few reports, the initial step of microbial hydrolysis of polyolefins is analogous to n-alkane resulting in hydroxyl group formation followed by further oxidation to generate aldehydes and ketones further into the corresponding acid; carboxylated hydrocarbon fragment can enter into beta-oxidation pathway due to

structural similarity, resulting in the depolymerization of two carbons in each cycle (Eyheraguibel et al. 2017).

Microbial bioremediation efficiency can be enhanced by making a suitable and competent consortium instead of using a single culture. The synergic metabolism between each microbial strain can increase the degradation rate of the complex substrate, and also they would contribute to the intermediary catabolic steps for other contributing strain (Varjani et al. 2015). However, this interdependence is difficult to measure experimentally, and understanding between biogeochemistry and metabolic processes of synthetic polymer utilization is still a void advance in the bioremediation area (Kujawinski 2011). The integration of technical and theoretical advances might help to clear our understanding of the microbial response and plastic mineralization mechanism (Dvořák et al. 2017).

1.5 Mechanism of Plastic Biodegradation

Recently, it had been proved that microbes have the potential to degrade complex structures like plastics and utilize them for growth and development. The principle of biodegradation is similar to that of other carbon-based substrates for microorganisms. Certainly, plastics act as carbon sources where microbes break their branched carbon chain into smaller and low molecular weight monomers or oligomer derivatives, which facilitate the intake of the released compounds in the cell to enter their carbon metabolism (Koutny et al. 2006). Wilkes and Aristilde (2017) suggested the surface brittlement and a decrease in hydrophobicity as a result of bond breakage due to photo-oxidation as well as CO_2 from polyethylene, when exposed to UV radiation; however, the addition of stabilizers protects the polymers from these abiotic disruptions.

The photochemical reaction involves the Norrish cleavage type (I and II) reaction, in which free radical and peroxide formation takes place, followed by chain cleavage and carbonyl and ester group formation (Wilkes and Aristilde 2017). Higher ambient temperatures have increased the rate of degradation because the activation energies for oxidative degradation are low for common plastics (Andrady 2015). The same phenomenon is also accountable for variable rates of weathering of differently colored plastics (Bucki et al. 2018). The microbial interaction over plastics and degradation entails a cluster of process due to its water insolubility and high molecular size. The process starts with the surface attachment to extracellular secretion for the chain depolymerization and fragments into smaller size, facilitating entry of the molecule inside the cell where most of the biochemical processes occur, and carbon dioxide and water are produced as end products (Pathak and Navneet 2017). In some cases, the extracellular enzyme acts only on the plastic surface where the internal structure remains intact, which also limits the degradation process (Kale et al. 2015).

Microorganisms flourish in various environmental conditions where many microbes render according to their surroundings as an outcome of adaptation.

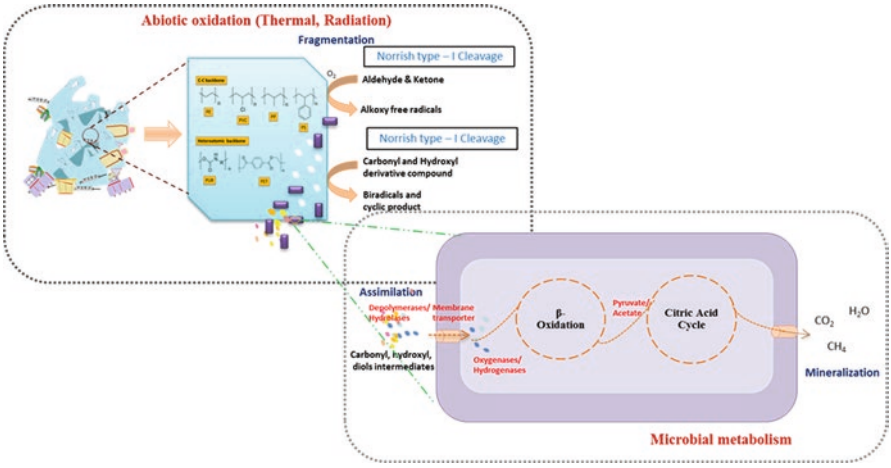

Fig. 1.3 Abiotic and microbial plastic degradation mechanism.

Amorphous regions of plastics are more exposed to microbial attack and attachment than internal structure and crystalline structures due to their structural and morphological complexity (Restrepo-Flórez et al. 2014). Microbial affinity and colonization over the polymer surface are the primary step in the degradation process followed by the beginning of the biochemical pathway for chain fragmentation and low molecular weight compound production for cell utilization (Fig. 1.3). The surface hydrophobicity of both plastic and microbe plays an important role in the colonization process (Pathak and Navneet 2017). The more hydrophobic microbes will attach more to the plastic surface and will reduce the hydrophobicity of the plastic (Orr et al. 2004). Surfactant production is also an important metabolic adaptation in the colonization process over hydrophobic surfaces.

The overall plastic biodegradation involved two key processes: first, reduction in molecular weight and, second, their oxidation (Restrepo-Flórez et al. 2014). The extracellular enzyme and physiological factors mediate the initial chain oxidation, although once the size of plastic molecules decreases to 10–20 carbon, olefins can enter the microbial cells and catabolize them through their metabolic pathway (Gautam et al. 2007). The formation and breakdown of bonds change the carbonyl bond index of the polymer, which may be due to the oxidoreductase enzyme activity of microorganisms (Wilkes and Aristilde 2017). However, there are very few researchers dedicated to the study of actual enzymes involved in these processes. Santo et al. (2013) proved the involvement of laccase enzymes in breaking down of large molecules and increased keto-carbonyl index during the incubation of polyethylene degradation.

Biotechnological tools can study the intermediate imprints of microbial-based degradations besides changes in the polymeric structures through analytical tools involving the appearance and disappearance of functional groups resulting from oxidation-reduction events during the degradation processes. Plastic is a

carbon-rich structure, so finding the exact mechanism of its mineralization through biological means would be revolutionary to the fermentation industry (Wierckx et al. 2015). In recent years, a number of microbes have been isolated and identified which are capable of degrading recalcitrant plastic material. They are potential candidates for the development of biocatalytic strategies for plastic recycling processes, by which valuable raw materials can be recovered and produced from the carbon in plastic in an environmentally friendly sustainable way (Urbanek et al. 2018). However, the effect of this technology on reducing waste is often compared with incineration and recycling methods.

Yoshida et al. (2016) reported two enzymes (polyethylene terephthalate and mono-2-hydroxyethyl terephthalate) involved in the process of converting polyethylene terephthalate into harmless products, mono-2-hydroxyethyl terephthalate, terephthalic acid, and ethylene glycol, where terephthalic acid is ingested by the cell through the terephthalic acid transporter, where it is catabolized by dehydrogenases and hydrogenases for cellular metabolism. Other investigations are also going on the transformation of waste plastic into alternative fuel oil or gas through catalytic pyrolysis; however, this method is proved to lack stable products and to be uneconomical (Panda et al. 2010). Since, research on biodegradation studies has progressed and enables researchers to work toward the unknown and unmapped side of the biodegradation mechanism. However, it is only possible if there is proper waste collection and separation of different types of plastic waste for further processing because depolymerized products of different plastic will vary. It will be an ecofriendly approach for reducing plastic waste as well as a solution to distressing situations due to plastic waste generation (Wei and Zimmermanm 2017).

1.6 Standards of Biodegradation Testing Methods

With rising plastic waste in the environment, several views and reports have been proposed for the biodegradation of plastics. However, many inadequacies in studies lack uniformity for productive output, necessitating the development of a directed standard testing method for biodegradation studies. Biodegradation studies are a broad and interdisciplinary area where chemistry, microbiology, biochemistry, molecular biology, bioinformatics, and ecology should be considered for building eminent parameters in the area.

Several microorganisms have been screened for plastic biodegradation using various analytical techniques such as molecular weight, topography, tensile strength, and gravimetric and functional group indexes. Current biodegradation analysis methods lack adequacy, such as biofilm formation, surface oxidation, or brittlement. Certainly, adherence of microorganisms indicates their affinity toward the substrate (Balasubramanian et al. 2010; Kumari et al. 2019); however, this does not necessarily indicate biodegradation. It could be abiotic factors that lead to structural and morphological changes but not biodegradation of microorganisms. Also, we cannot be sure about the survival of microbes for the complete duration of the test. Initially,

biodegradation evaluation focused on CO_2 and CH_4 evolution, weight loss, and molecular weight changes (Pathak and Navneet 2017). The biased experimental designs can misinterpret biodegradation output, such as modified (starch or vegetable oil blending) or treated (thermal or UV pretreatment) plastic materials, studying the same polymer from different sources with varying standard properties, using different culture conditions and media, lack of overall understanding of biodegradation end products, limited information about enzymes involved in biodegradation and genes encoding them, and lack of any definitive biodegradation mechanism and implication of study in natural environments (Joutey et al. 2013). Also, regulation of standard testing methods to account for the adverse effects of plastic leachate products in the food chain and mineralization processes in the ecosystem is needed (Lithner et al. 2011; Pathak and Navneet 2017).

Few guidelines and regulations have been made for bioremediation research in a defined way for its productive implications (Table 1.3). The world international government forum engaged different countries in developing regulations in different aspects where, in year 1981, the Economic Co-operation and Development and European Committee for Standardisation (1996) issued guidelines for standardized biodegradation evaluation methods for plastic wastes, which include microbial respiration measurement for analytical determination of biodegradation and included tests for characterizing the stability of plastic (Vroblesky 2001). They are likely to be applied in countries where they were developed and a wide scope for the development of consistency in international standards (Table 1.3). The improvement in experimental methodologies is essential to simplify the development of new biodegradability standards of plastics for simulating in natural environments to maintain method and output consistency (Briassoulis and Dejean 2010).

Field practices can only provide the true measure and throughput for reliable biodegradation research output. However, the results of exposure to the environment are likely to differ substantially from the laboratory scale study and to one another's environment. The degradation time scale may vary from month to year in the environment than in laboratory experiments. However, there are challenges and questions that have been addressed for new perspectives.

1.7 Biodegradable Polymers

Concerning with the non-degradability of petrochemical-based plastics, the development of biodegradable polymers has been emphasized as an alternative (Badia et al. 2017). The increasing recalcitrant plastic pollutants and depleting the petroleum fraction; bio-based polymers have attracted attention and are considered as a solution for environmental sustainability and conservation (Elsawy et al. 2017; Panwar et al. 2011). Biodegradable polymers are considered bio-based or vice versa, whereas biodegradable polymers decompose into CO_2, H_2O, and inorganic compounds, and bio-based polymers are derived from biological sources that are obviously biodegradable (i.e., polylactate, polybutyrate adipate terephthalate)

Table 1.3 International standards defined for plastic biodegradation evaluation methods

S. No.	Regulation	Organization	Method	Experiment
1.	ASTM	American Society For Testing and Materials	D6954: 18	Exposing and testing plastics that degrade in the environment by a combination of oxidation and biodegradation
2.	BSI	British Standards Institution	BS EN ISO 10210: 2017	Sample preparation method for biodegradation testing of plastic materials
3.	ISO	International Organization For Standardization	ISO 846: 1997	Evaluation of the action of microorganisms on plastics
			ISO 17556: 2012	Aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved
			ISO 18830: 2016	Aerobic biodegradation of non-floating plastic materials in a seawater/sandy sediment interface by measuring the oxygen demand in closed respirometer
			ISO 14853: 2016	Anaerobic biodegradation of plastic materials in an aqueous system by measuring the biogas production
			ISO 19679: 2016	Aerobic biodegradation of non-floating plastic materials in a seawater/sediment interface by analysis of evolved carbon dioxide
4.	AFNOR	Ente Nazionale Italiano Di Unificazione (UNI)	UNI 11462: 2012	Plastic materials biodegradable in soil – types, requirements, and test methods
			UNI EN ISO 14855-1: 2013	Aerobic biodegradability of plastic materials under controlled composting conditions by analysis of evolved carbon dioxide – part 1: general method
5.	NSAI	National Standards Authority Of Ireland	I.S. EN ISO 10210: 2017	Sample preparation for biodegradation testing of plastic materials
			I.S. EN ISO 17556: 2012	Aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved
			I.S. EN ISO 14855-1: 2012	Aerobic biodegradability of plastic materials under controlled composting conditions by analysis of evolved carbon dioxide – part 1: general method
			I.S. EN ISO 14855-2: 2018	Aerobic biodegradability of plastic materials under controlled composting conditions by analysis of evolved carbon dioxide – part 2: gravimetric measurement of carbon dioxide evolved in a laboratory-scale test

(Luckachan and Pillai 2011; Sudesh 2013; Murariu and Dubois 2016). Bio-based plastics have been more promoted in the preceding years by the public due to environmental sustainability, climate change, and the exhaustion of fossil fuels (Shen et al. 2010). In general, polymers derived from biological sources are considered to be capable of breaking down into nontoxic and entering the biogeochemical cycling of ecosystems after a certain period. Different strategies have been applied for producing bio-based plastics such as the bacterial product polyhydroxyalkanoate, polymeric blends with lipids and polysaccharides, using agricultural feedstock for producing polyhydroxyalkanoate, and use of biofuel instead of conventional petroleum fraction for polymer synthesis (Ramírez et al. 2017; Kalia et al. 2015). Polyhydroxyalkanoate is a well-known biodegradable polymer that is produced from bacteria. Moreover, many research on polyhydroxyalkanoate production is going on to reduce the production cost through application of genetically modified bacterial strains or by incorporating polyhydroxyalkanoate producing gene in plants for more yield and production (Pagliano et al. 2017; Lee et al. 2011). Reddy and Yang (2011) developed a biodegradable composite of soyprotein-jute biocomposites. Several parameters have been reported to influence the degradation behavior of biodegradable polymers; the most important factors are moisture sensitivity and stability, which is also a limitation to broad applications (Farah et al. 2016).

Still the limited application and high production cost of biopolymers are making unsatisfactory to compete with synthetic plastics and not getting much attention in the market. Other approaches are also in trial, such as blending of natural materials like starch, protein, lactic acid, cellulose, or collagen with conventional one which will provide stability to the material and more application opportunities as well as enhanced degradation rates (Leja and Lewandowicz 2010; Luckachan and Pillai 2011). Table 1.4 shows a few examples of various biopolymer derived with different strategies to replace plastics. Additionally, plasticizers are used to improve durability. This provides a basis for researchers to look for the ultimate alternative to conventional plastic. However, complete degradation of these polymers will be cynical, and problems will go in the same way.

Table 1.4 Various environmentally friendly biopolymer

Source	Biopolymer	References
Biomass	Soyprotein–jute fiber composites	Reddy and Yang (2011)
Fermentation product	Polylactic acid	Elsawy et al. (2017)
	Polyhydroxyalkanoates	Bhatia et al. (2019)
Biopolymer blend	Poly(butylene adipate co-terephthalate)	Wang et al. (2016)
	Polyepoxidized soybean oil-co-decamethylene diamine	Wang et al. (2012)
	Soybean oil-based poly(vinyl chloride)	Chen et al. (2017)

1.8 Recent Drifts in Microbial Degradation Research

Research on plastic biodegradation is going from half a century, while root information about plastic mineralization and utilization by microorganisms is beyond the wall. The standard design and knowledge about biochemical routes for whole-cell biocatalysis can provide a visionary model of new dimensions for engineering microbes for better bioremediation prospects. Continuous development in research technology has provided an endless opportunity to endure and uncover veiled facts (Kawai et al. 2019).

Few concomitant queries for the plastic utilizing ability of microbes, their interaction with the hydrophobic surface of the substrate, to endure and grow in diverse environmental conditions are still unclear. Technological and conceptual advancement will drive a transitional bottleneck to new insights in the bioremediation field. Today, the era of genomics and metabolomics techniques is playing a significant role in resolving many research mysteries in the broad sector of science. It can also uncover the wider opportunities for the exploitation of microbes that represent an important asset for maintaining a sustainable environment. Emerging genomic and high-throughput metabolomic technologies are the way out solution to study the whole microbial system for their bioremediation potential. The availability of rich bioinformatics databases and existing computational tools enables new insights to aid in data mining and pathway prediction (Dvořák et al. 2017; de Lorenzo 2018). The challenge ahead is to identify the pathways which are expressed in different environmental conditions (Kujawinski 2011). Genomic evidence provides a schematic overview of all possible metabolic pathways in individual microorganisms or in complex consortia (Guo et al. 2017). The RNA expression (transcriptomics), metabolic (metabolomic), and protein (proteomic) profiling, the so-called omic techniques, will build on potential process-oriented informative (Fig. 1.4) as well as their combination with system biology, which is a computational and mathematical model of all the data generated that can provide a relative prediction of cellular

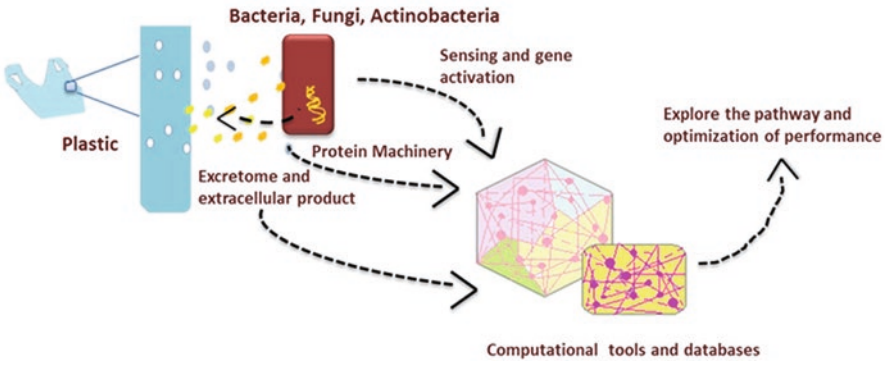

Fig. 1.4 Illustration of microbial action on plastic and degradation pathway prediction by using various tools

metabolism and genetic background (Swartz 2018; Holmes et al. 2019). The omic technology and system biology can outline the regulatory processes and genes for further improvement or optimization of microbial degradation processes by engineering host cells (Shah et al. 2011; Dvořák et al. 2017; Gravouil et al. 2017).

The research on biodegradation studies advances and enables researchers to work toward the unknown and unmapped side of the biodegradation mechanism (Yoshida et al. 2016; Gravouil et al. 2017; Yang et al. 2014). By the development of molecular techniques, it is possible to study the molecules involved and released during the polymer utilization by the microbe, while the actual step involved in the degradation processes is uncovered. Imprinting of the microbial-based degradation and changes in the polymeric structures through analytical tools involves the appearance and disappearance of functional groups resulting from oxidation-reduction events during the degradation processes. Wilkes and Aristilde (2017) outlined the polyethylene biodegradation pathway; however, the actual microbial degradation path for plastic is still unclear. There has been no practical application of biodegradation on a commercial scale yet. However, extensive research needs to be conducted to explore the enormous metabolic potential of microorganisms and bring it up to the translational level.

In one outlook, recalcitrant plastic waste and their degraded intermediates can be utilized as next-generation carbon sources (Wierckx et al. 2015). In the future, we need to isolate and identify novel microbes and genes with the help of advances in sequencing technology and new tools for monitoring microbial metabolism for hydrocarbon substrates (Austin and Callaghan 2013).

1.9 Conclusion

The increasing and spreading of plastic waste in the environment needs to the application of bioremediation technology for reduction. We are at the edge of the bioremediation revolution, where understanding the microbes and molecule interactions might be the basis for the global carbon cycle. Environmental pollution treatment by means of microorganisms would be a promising tool; however, a combination of various approaches can make it possible to implicate and engineer the candidate organism to optimize the enzyme activity, metabolic pathways, and growth conditions for the biodegradation processes of plastics. Many microorganisms from different sources have been harnessed for biodegradation studies of different types of plastics, while the degradation efficiency of these microbes is a halt for large-scale applications. Perhaps, there is also variation in biodegradation testing methods that makes the inconsistency and inadequacy in the field and commercial trials. Knowledge of the rate and end product of the plastic contaminant biotransformations before field practice is mandatory. The significant development of omic technology and bioinformatics databases will help in understanding the essential roles of microbial metabolism in the biotransformation of plastics. Our current

understanding and knowledge can deduce tentative microbial plastic degradation systems, which can be explored further for more efficiency.

Improper waste management framework practice for aggregation of plastic waste and separation of different types of plastic for further processing is also one of the major flaws to control increased synthetic waste in the environment. It is the responsibility of both the common public and researchers to change the outlook toward the problem. The epidemic increase of plastics in the environment cannot be controlled by individual sectors, the mutual action of the public, industries, scientists, and policymakers by minimizing the use of plastics in daily life, increasing recycling capability, development of bio-based feedstocks for plastic production, and strict regulation for waste aggregation and collection systems.

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Chapter 2
Biodegradation and Bioremediation of *S*-Triazine Herbicides

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Contents

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Abstract Pesticides have emerged as an integral tool of the farming activities and are used extensively to meet the increasing demand for food and feed. About 99% of the applied pesticides get accumulated in the nontarget organisms and environment. S-triazine herbicides have been classified as possible human carcinogens. Of these, atrazine was mostly used as it increases yield up to 50% based on crop and the most studied for toxicity and degradation. These pesticides are slowly degraded, and persistence leads to accumulation in soil or migrate to water bodies posing a severe threat to human and environment. Atrazine and its metabolites are frequently detected in surface water and ground water at concentrations exceeding the safety levels. We reviewed the biodegradation of s-triazine herbicides by microorganism, plants, and their degradation pathways. It was noted that atrazine degrading genes are widely distributed among the bacteria, but most of the bacterial strains do not contain all the genes required for atrazine mineralisation. Atrazine mineralisation appears to be more common in soils by microbial consortia than individual species. Certain bacteria including *Arthrobacter* sp. SC-JAK2 can degrade atrazine of above 1 g L^{-1} concentration which is far above the reported atrazine contaminant concentration in soil and water. Several reports concluded that excellent atrazine degraders in laboratory media, fail to do that in the complex natural environmental conditions that are suboptimal for growth or repress the synthesis of enzymes involved in the degradation pathway. Biostimulation and bioaugmentation studies showed rapid biodegradation of atrazine in contaminated sites. Major advances in the biodegradation of *s*-triazine-contaminated sites include the usage of genetically modified or engineered microorganisms, enzymatic bioremediation, and use of nanomaterials. With the help of advanced molecular and physiological approaches, it is possible to monitor the bioremediation and microbial community development in the atrazine-contaminated soil.

Keywords Atrazine · *S*-Triazine · Bioaugmentation · Biodegradation · Bioremediation · Biostimulation · Contaminant · Environment · Herbicides · Pesticides

2.1 Introduction

To meet the global requirement of food and fuel to some extent, pesticides are being used extensively in agriculture. Less than 0.1% of the applied pesticides reach the target organism, and the remainder gets deposited in soil and nontarget organisms or move into nearby water streams and lakes by leaching and agricultural runoff (Pimentel and Levitan 1986). These pesticides cause contamination of the environment and adversely affect the nontarget organisms and plants because of their persistence in the soil and water bodies. The persistence of pesticides in the soil and water mainly depend on chemical stability, solubility in water, soil physicochemical properties, climatic conditions, soil microbial activity, and leaching. Pollution of soil and water with pesticides and their toxic metabolites have become a major

environmental concern in the twenty-first century. Hence, the obliteration of persistent pesticides is essential to their sustained use.

2.1.1 S-*Triazine Herbicides*

Symmetrical triazine (*s*-triazine) relates to a large family of herbicides widely used worldwide to control broadleaf weeds and annual grasses in various plantations, residential lawns, and golf courses. The first triazine, chlorazine, was discovered in 1952 at J.R. Geigy Ltd. in Switzerland. Later in 1956, atrazine and simazine were discovered. The general structure of *s*-triazine herbicides is shown in Fig. 2.1. Side chains of triazine ring (X, R_1, and R_2) of commonly used triazine pesticides and their half-life in soil, applications in crops, as well as WHO classification are given in Table 2.1. Examples of *s*-triazines herbicides are chloro-*s*-triazines (atrazine, simazine, propazine, and cyanazine), the thiomethyl-*s*-triazines (ametryn, prometryn, terbutryn), and the methoxy-*s*-triazine (prometon). Cyanuric chloride (trichloro-1, 3, 5-triazine) is the basic for the production of several *s*-triazine herbicides including atrazine and simazine. Triazines are taken up into the plant roots, distributed throughout the plant via xylem, and act by interrupting photosynthesis in leaves specifically inhibiting the photosystem II. The effectiveness of triazines is dependent on several parameters including soil structure, moisture content, organic matter content, particle size distribution, and mode of application. Major advantages of using triazines are that it offers application flexibility and facility to mix with other herbicides for broad-spectrum weed control. They provide exceptional residual preemergence as well as early postemergence weed control. This enable farmers to use no-till and conservation tillage systems that minimise soil erosion by more than 50%. Triazine herbicides played a significant role in the adoption of conservation tillage, which significantly reduced fuel usage since fewer tillage trips are made across the field. Conservation tillage systems conserve soil moisture, increase the soil organic matter, and also dramatically decrease the water runoff and increase water infiltration. Minimizing soil erosion and water runoff will benefit the aquatic ecosystem. Further, these triazine herbicides paved way for the increased yield of food and feed in lesser space.

Only a fraction like less than 1% of the applied herbicide reaches the site of action within the plants. The loss is due to volatilisation, adsorption to soil, leaching

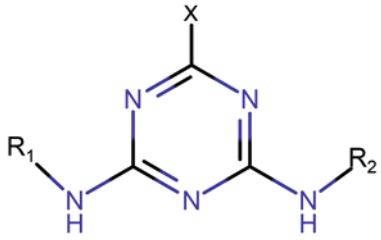

Fig. 2.1 General chemical structure of *s*-triazine pesticides (X, R_1, and R_2 are the side chains of triazine ring)

Table 2.1 List of *s*-triazine pesticides

s-Triazine herbicides	X	R_1	R_2	Soil half-life (days)	Applications	WHO classification
Atrazine	-Cl	$-NHC_2H_5$	$-NHC_3H_7$ (iso)	14–150	Corn, sorghum, sugarcane, pineapple, conifers, forestry	Slightly hazardous
Atratone	$-OCH_3$	$-NHC_2H_5$	$-NHC_3H_7$ (iso)	30	Non-agricultural areas, sugarcane, corn	Obsolete substance
Ametryn	$-SCH_3$	$-NHC_2H_5$	$-NHC_3H_7$ (iso)	37–250	Sugarcane, corn, pineapple	Moderately hazardous
Cyanazine	-Cl	$-NHC(CN)(CH_3)_2$	$-NHC_2H_5$	12–25	Vegetables, onions, potatoes, sweetcorn	Moderately hazardous
Desmetryn	$-SCH_3$	$-NH_2CH_3$	$-NHC_3H_7$ (iso)	9–50	Brassicas, onions and leeks, fodder rape	Slightly hazardous
Dimethametryn	$-SCH_3$	$-NHC_2H_5$	$-NHCH(CH_3)C_3H_7$ (iso)	37–250	Sugarcane, corn, pineapple	Slightly hazardous
Prometryn	$-SCH_3$	$-NHC_3H_7$ (iso)	$-NHC_3H_7$ (iso)	41–60	Cotton, celery, dill, potatoes, sunflowers, carrots, peanuts	Slightly hazardous
Prometone	$-OCH_3$	$-NHC_3H_7$ (iso)	$-NHC_3H_7$ (iso)	500	Non-cropland	Slightly hazardous
Propazine	-Cl	$-NHC_3H_7$ (iso)	$-NHC_3H_7$ (iso)	45–131	Corn, sorghum, carrots, fennel, ornamentals, greenhouse use	Unlikely to present an acute hazard
Simazine	-Cl	$-NHC_2H_5$	$-NHC_2H_5$	60–102	Corn, fruit and nut crops	Unlikely to present an acute hazard
Simetryn	$-SCH_3$	$-NHC_2H_5$	$-NHC_2H_5$	60	Rice, corn, bean, pea, cereals, cotton	Slightly hazardous
Terbutryn	$-SCH_3$	$-NHC_2H_5$	$-NHC(CH_3)_3$	14–74	Sugarcane, cereal, sorghum, sunflowers, peas, potatoes	Slightly hazardous

(continued)

Table 2.1 (continued)

s-Triazine herbicides	X	R_1	R_2	Soil half-life (days)	Applications	WHO classification
Trietazine	-Cl	$-N(C_2H_5)_2$	$-NHC_2H_5$	60	Potatoes, legumes, bananas, citrus, coffee, maize, sugarcane, tea, tobacco	Slightly hazardous
Terbuthylazine	-Cl	$-NHC_2H_5$	$-NHC(CH_3)_3$	22–60	Corn, sorghum, grape	Slightly hazardous

Where X, R_1 and R_2 = side chains of triazine ring (shown in Fig. 2.1)

by rainfall, photochemical degradation by sunlight, microbiological degradation by soil microorganisms, chemical degradation by soil constituents, and thermal degradation. *S*-triazines are toxic compounds and have been classified as possible human carcinogens. Simetryn, one of the major methylthio-*s*-triazine herbicides used in paddy fields, inhibits algal growth. Chlorinated triazine class of pesticides show common neuroendocrine mechanism of toxicity resulting in both reproductive and developmental concerns. The toxicity of these compounds has promoted research for their degradation. Atrazine is the most studied chlorinated triazine herbicide for toxicity and degradation.

2.1.2 Atrazine

Atrazine is one of the most widely used herbicides. Atrazine is very effective against a wide range of weeds and less expensive when compared to the alternative products. Atrazine inhibits D-1 quinone binding involved in photosystem II. Atrazine increases yield of about 6–50% based on crop. Despite the high agricultural yield, there is a huge concern for the continued use of atrazine in several parts of the world. For three decades from its discovery, microbial degradation was not observed as the triazine ring contains no available electrons for aerobic biodegradation. For microbial degradation, the only available energy source in atrazine is the ethyl and isopropyl side chains attached to the triazine ring. However, atrazine was converted by nonspecific monooxygenases to desethylatrazine and desisopropylatrazine. Small amount of hydroxyatrazine was formed by chemical processes. Atrazine and its metabolites are frequently detected in surface water and ground water at concentrations exceeding the safety levels. Atrazine is believed to cause endocrine disruption; carcinogenic effects including non-Hodgkin's lymphoma, ovarian cancer, colon cancer, leukaemia, multiple myeloma, reduced sperm quality in humans (IARC 1999; Rusiecki et al. 2004), cancer, delayed reproductive development in

rats, and male hermaphroditism in amphibians; and negative effect on aquatic organisms particularly in combination with other pesticides.

In anaerobic aquatic environment, atrazine's overall half-life, water half-life, and sediment half-life were given as 608, 578, and 330 days, respectively. While in terrestrial environment, half-life of atrazine may range from 13–261 days (US-EPA 2006). Atrazine dealkylation metabolites, such as deethylatrazine and deisopropylatrazine, are also regulated compounds and may pose health risks. Massive application, high mobility, and persistence are the major reasons for the frequent detection of atrazine and its metabolites in surface and ground water at concentrations well above the legal limits globally. European Union banned atrazine use in October 2003 but still in use in many parts of the world including the United States and India. However, Environmental Protection Agency has set the maximum containment level for atrazine in drinking water at 3 ppb. Triazine herbicides are persisted in the soil for 3–12 months and are slowly degraded by biological, chemical, and physical processes. This persistence period leads to accumulation in soil and water bodies posing a serious threat to human and environment. Therefore, utmost priority should be given to develop effective technologies for detoxification and/or removal of triazine pesticides and their metabolites. However, the metabolites of atrazine including hydroxyatrazine is less acutely toxic than the parent atrazine.

2.2 Biodegradation

2.2.1 Biodegradation of S-*Triazine Herbicides by Bacteria*

Pseudomonas sp. strain ADP was the first isolated atrazine-mineralizing strain. Many other bacteria are found to degrade atrazine as shown in Table 2.2. Atrazine mineralisation by microbial consortia appears to be more common in soils than individual species as most of the bacterial strains do not contain all the genes required for atrazine mineralisation (Billet et al. 2019; Kolic et al. 2007; Smith et al. 2005). Bacteria use atrazine primarily as a nitrogen source. Satsuma (2010) reported that a newly isolated *Nocardioides* species strain DN36 not only mineralised simetryn, atrazine, and simazine but also transformed propazine, ametryn, prometryn, dimethametryn, atraton, simeton, and prometon.

Degradation Pathway

Triazine mineralisation is more or less similar to atrazine mineralisation and is achieved in two stages. In the first stage, atrazine is converted to cyanuric acid (2,4,6-trihydroxy-1,3,5-triazine) by dehalogenation and dealkylation of side chains. Conversion of atrazine to cyanuric acid takes place via one of the three pathways as shown in Fig. 2.2. P-1 is the hydrolytic pathway commonly found in many bacteria

Table 2.2 List of bacterial genera capable of degrading various triazine herbicides

Pesticide	Bacteria	References
Atrazine	*Acinetobacter* sp.	Singh et al. (2004a)
	Aerobacterium sp.	Vargha et al. (2005)
	Agrobacterium sp.	Devers et al. (2007)
	Arthrobacter sp.	Shapir et al. (2005a)
	Bacillus sp.	Vargha et al. (2005)
	Chelatobacter sp.	Rousseaux et al. (2001)
	Citricoccus sp.	Yang et al. (2018)
	Deinococcus sp.	Vargha et al. (2005)
	Clavibacter sp.	De Souza et al. (1998)
	Delftia sp.	Vargha et al. (2005)
	Microbacterium sp.	Vargha et al. (2005)
	Micrococcus sp.	Vargha et al. (2005)
	Nocardioides sp.	Piutti et al. (2003)
	Polaromonas sp.	Devers et al. (2007)
	Pseudaminobacter sp.	Topp et al. (2000)
	Pseudomonas sp.	Mandelbaum et al. (1995)
	Ralstonia sp.	Radosevich et al. (1995)
	Rhizobium sp.	Bouquard et al. (1997)
	Rhodococcus sp.	Behki et al. (1993)
	Sinorhizobium sp.	Devers et al. (2007)
Ametryn	*Agrobacterium* sp.	Moscinski et al. (1996)
Cyanazine	*Agrobacterium* sp.	Moscinski et al. (1996)
	Rhodococcus sp.	Behki (1993)
Prometon	*Agrobacterium* sp.	Moscinski et al. (1996)
Prometryn	*Bacillus* sp.	Mizrachi (1994)
	Leucobacter sp.	Liu et al. (2018)
	Pseudomonas sp.	Grossenbacher (1986)
Propazine	*Rhodococcus* sp.	Behki (1993)
Simazine	*Acinetobacter* sp.	Feakin et al. (1995)
	Agrobacterium sp.	Liao and Xie (2008)
	Klebsiella sp.	Sánchez et al. (2005)
	Rhodococcus sp.	Behki (1993)
	Moraxella (Branhamella) sp.	Kodama et al. (2001)
	Pseudomonas sp.	Hernandez et al. (2008)
Simetryn	*Bacillus* sp.	Mizrachi (1994)

Note: Gram-positive bacteria have the ability to degrade more than one *s*-triazine pesticide

catalysed by atrazine chlorohydrolase (AtzA) or triazine hydrolase (TrzN), hydroxyatrazine N-ethylaminohydrolase (AtzB) (BoundyMills et al. 1997), and N-isopropylammelide aminohydrolase (AtzC) (Sadowsky et al. 1998). In Gram-negative bacteria, AtzA is responsible for dechlorination of atrazine (deSouza et al. 1996). TrzN of Gram-positive bacteria showed broad-spectrum activity, not only dehalogenation but also dislodges azido, cyano, S-alkyl, and O-alkyl substituents of

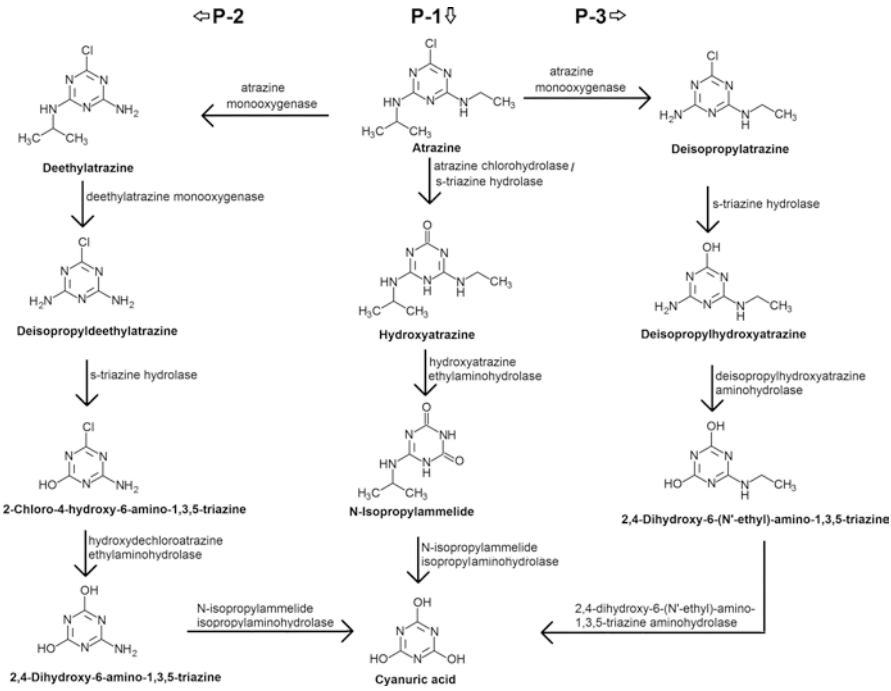

Fig. 2.2 Atrazine degradative pathways (P1, P2, and P3) showing conversion of atrazine to cyanuric acid

s-triazine herbicides (Topp et al. 2000). P-2 and P-3 are oxidative-hydrolytic pathways involving initial oxidative N-dealkylation of atrazine to deethylatrazine or deisopropylatrazine respectively by nonspecific monooxygenases (Devers et al. 2004). These products are dealkylated again to deisopropyldeethylatrazine, which is converted to cyanuric acid by hydrolytic dechlorination, deamination, and/or dealkylation (Govantes et al. 2009). Atrazine degradation via these routes is mostly reported by a consortium rather than individual bacteria and is less common. In the second stage, hydrolytic cleavage of the s-triazine ring of cyanuric acid and subsequently hydrolysis of biuret and allophanate occur to yield ammonia and carbon dioxide (Fig. 2.3). In most of the atrazine mineralising bacteria, these enzymes are encoded by the *atzDEF* operon (Fruchey et al. 2003; Karns 1999; Shapir et al. 2005b). Homologues to AtzD (TrzD) and AtzF (TrzF) perform the equivalent reactions in other bacteria with small differences in substrate affinity and specificity (Rousseaux et al. 2001; Shapir et al. 2006). Atrazine will be biodegraded to cyanuric acid by one of the above three pathways.

Rhodococcus sp. strain FJ1117YT degrades the methylthio-*s*-triazines such as simetryn, ametryn, desmetryn, dimethametryn, and prometryn when supplied as the sole sulphur source. The biodegradation pathway of simetryn involves the formation of methylsulfinyl analogue as the first metabolite followed by methylsulfonyl

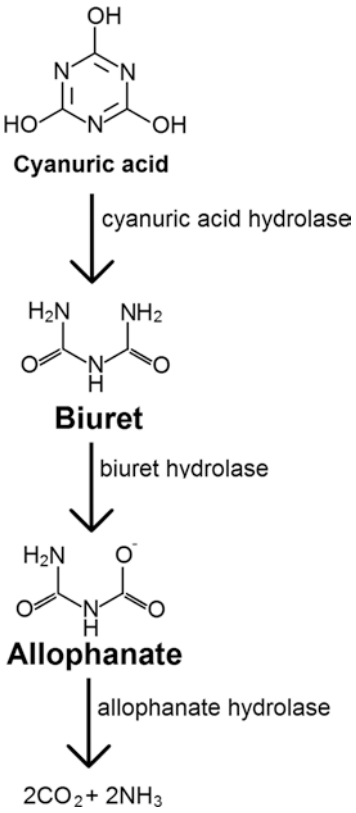

Fig. 2.3 Cyanuric acid degradative pathway showing conversion of cyanuric acid to carbon dioxide and ammonia

intermediate and the hydroxy analogues. The methylthio group of methylthio-*s*-triazines was progressively oxidised and hydrolysed (Fujii et al. 2007). Simazine degradation occurs via two pathways yielding either 2-hydroxysimazine or desethylsimazine. Organic or inorganic nitrogen sources stimulated N5C cell growth, but had little effect on the simazine degradation rate. Some of the bacteria use prometryn or ametryn as the sole source of sulphur for growth (Cook and Hütter 1982). Methylthio-*s*-triazines were transformed to their hydroxy compounds by whole cells and cell extracts of *Nocardioides* sp. strain C190 (Topp et al. 2000) and *Clavibacter michiganensis* strain ATZ1 (Seffernick et al. 2000). Recombinant TrzN from *Arthrobacter aurescens* strain TC1 rapidly hydrolyses ametryn and methylsufinyl ametryn to hydroxyametryn (Shapir et al. 2005a).

S-Triazine-Degrading Proteins

AtzA is a homohexamer of the amidohydrolase superfamily which contains one essential Fe^{2+} per monomer. AtzA hexamer is a trimer of dimers with a molecular weight of 315 kDa. AtzA gene has been proposed to be evolved from the TriA gene

(from *Pseudomonas* sp. strain NRRL B-12227) with only nine amino acid substitutions in response to atrazine induction. Despite of 98% sequence similarity, they are functionally different. AtzA is a dechlorinase with no deaminase activity, while TriA is a deaminase with low dechlorinase activity. TrzN is a zinc-dependent amidohydrolase which is ~25% identical to atzA (Mulbry et al. 2002). TrzN is a dimer containing a single Zn^{2+} bound in each active site. Both AtzB and AtzC (N-isopropylammelide aminohydrolase) have a zinc metal centre in the active site. Shapir et al. (2002) reported that molecular weight of AtzC holoenzyme is 174,000 and has a subunit size of 44,938 kDa. The activity of metal-depleted AtzC can be restored with Zn(II), Fe(II), Co(II), Mn(II), and Ni(II) salts. AtzD enzyme is a member of a family of ring-opening amidases. Apart from those enzymes discussed earlier, some other enzymes are reported to involve in the mineralisation of atrazine in few organisms. These include *Rhodococcus* sp. N186/21 cytochrome P450 (Nagy et al. 1995). Smith et al. (2005) reported that *Nocardia* converted hydroxyatrazine to N-ethylammelide via an unidentified gene product.

S-Triazine-Degrading Genes

Triazine-degrading genes may be located on large plasmids or on the bacterial chromosome (Devers et al. 2007). The atzABCDEF gene composition was found only in few bacterial strains including *Pseudomonas* sp. ADP and *Agrobacterium* sp. NEA-D and is located on a unique plasmid of 110 kb for ADP (pADP1 plasmid) and 137 kb for NEA-D. Atrazine mineralisation was well studied using *Pseudomonas* sp. ADP. AtzABC genes are dispersed in an unstable region and flanked by insertion elements with high homology to the known transposable DNA elements, IS1071 and IS801. The rearrangements result in the stochastic loss of one, two, or all three atz genes. In the absence of atrazine selection pressure, atzB can be easily lost as in *Aminobacter ciceronei* strain C147 formerly *Pseudaminobacter* sp. (Topp et al. 2000). The genes encoding atzDEF are clustered in the atzDEF operon, which is located in a stable region of pADP-1. Adaptation of soil microflora to atrazine degradation or mineralization may rely on horizontal gene transfer and repeated exposure. Atrazine mineralization greatly depends on regulatory phenomena in response to nitrogen limitation and transcriptional activation by LysR-transcriptional regulators. Devers et al. (2007) reported the presence of TrzN gene in Gram-negative bacteria such as *Sinorhizobium* sp. and *Polaromonas* sp.

Recombinants and Formulations

Genetically engineered microorganisms overexpressing catabolic genes considerably amplify the degradation in heavily atrazine-contaminated soils. Strong et al. (2000) employed transgenic AtzA-expressing *E. coli* to remove residual atrazine contamination in situ of soil contaminated with 29 g L^{-1} atrazine. Benson et al. (2018) observed superior biodegradation of atrazine by recombinant *E. coli*-expressing atrazine

chlorohydrolase encapsulated in organically modified silica gel. They reported that atrazine biodegradation is highly dependent on the adsorption.

2.2.2 *Biodegradation of* S-*Triazine Herbicides by Fungus*

Several soil fungi including *Aspergillus fumigatus*, *A. flavipes*, *A. ustus*, *Fusarium oxysporum*, *F. roseum*, *F. moniliforme*, *Rhizopus stolonifer*, *Penicillium decumbens*, *P. luteum*, *P. janthinellum*, *P. rugulosum*, and *Trichoderma viride* are reported to degrade atrazine by N-dealkylation of either alkylamino groups. They were unable to cleave the triazine ring. Although dealkylation is a pathway in majority of the fungal strains, formation of hydroxyatrazine was also observed in few fungal species such as *P. luteum* (Kaufman and Blake 1970). Other atrazine-degrading fungal strains include white rot fungus *Phanerochaete chrysosporium* (Mougin et al. 1994) and *Pleurotus pulmonarius* (Masaphy et al. 1993). Donnelly et al. (1993) studied the atrazine degradation efficiency of *Hymenoscyphus ericae*, *Oidiodendron griseum*, *Trappea darkeri*, and *Rhizopogon vinicolor* and reported that with increase in nitrogen concentration results in increased herbicide degradation. *Penicillium steckii* DS6F is the first simazine-degrading fungus ever reported (Kodama et al. 2001). Szewczyk et al. (2018) reported the degradation of the ametryn by entomopathogenic fungi. *Metarhizium brunneum* leads to formation of 2-hydroxy atrazine, ethyl hydroxylated ametryn, S-demethylated ametryn, and deethylametryn.

2.2.3 *Biodegradation of* S-*Triazine Herbicides by Plants*

In plants, three metabolic pathways are involved in atrazine transformation. The major pathway of atrazine detoxification in some resistant weeds is glutathione conjugation in which the glutathione *S*-transferase displaces chlorine atom at 2-carbon atom of atrazine (Lamoureux et al. 1970). The second mechanism is hydrolysis where the chlorine atom in atrazine is replaced with a hydroxyl group. Resistance of corn to atrazine and simazine was primarily attributed to 2- hydroxylation pathway (Hamilton and Moreland 1962). The third pathway is N-dealkylation, in which cytochrome P450 monooxygenases remove the ethylamino and isopropyl amino side chains. In pea and resistant sorghum, only the N-dealkylation pathway was performed in which atrazine is degraded to desethylatrazine and desisopropylatrazine. The first instance of atrazine uptake and degradation by aboveground plant biomass was shown in poplar trees (Burken and Schnoor 1997). In poplar trees, corn (*Zea mays* L.), and sorghum (*Sorghum vulgare* Pers.), atrazine metabolism occurs via 2-hydroxylation and N-dealkylation pathways (Shimabukuro 1967). Plant root exudates influence the atrazine degradation through the enhancement of microbial activity. Atrazine-contaminated soils planted with *Pennisetum clandestinum* showed faster atrazine degradation than in unplanted soil (Singh et al. 2004b). Rhizosphere

soils from *Kochia scoparia* and maize plants showed to accelerate mineralization of atrazine (Perkovich et al. 1996; Piutti et al. 2002). Wang et al. (2012) used a hydroponic system to evaluate the potential of three emergent hydrophytes, *Iris pseudacorus*, *Lythrum salicaria*, *and Acorus calamus* for atrazine removal and uptake. Schmidt et al. (2008) studied the biconversion of [^{14}C] atrazine to hydroxyatrazine and dealkylated products (de-ethyl-, deisopropyl- and de-ethyl-deisopropylatrazine) in heteroirophic cell-suspension cultures of soyabean (*Giycine max* L. Merr), carrot (*Daucus caroia*), purple foxglove (*Digitalis purpurea*), corn cockle (*Agrostemma githago*), wheat (*Tritician aestivum*), and thorn-apple (*Datura stramonium*).

2.2.4 Abiotic Degradation of S*-Triazine Herbicides*

Several physicochemical methods are proposed for cleaning of atrazine from contaminated soils, water, and wastewater. These techniques include incineration, reverse osmosis, electrodialysis, thermal absorption, ultraviolet, peroxides, and metal oxides. Various adsorbents including hypercrosslinked polymers (Streat and Horner 2000), zeolites, and organoclays (Bottero et al. 1994) have been studied for the removal of atrazine. Chemical methods used for atrazine degradation are photolysis, hydrolysis, dehalogenation, and oxygenation. Chemical hydrolysis of atrazine produces hydroxyatrazine in strongly acidic or basic solutions. These technologies are expensive and also release toxic by-products, which require further treatments. Atrazine degradation is negligible by sunlight, i.e. direct photolysis and result in the formation of hydroxyatrazine and dealkylated products of hydroxyatrazine. Photosensitisers such as dissolved organic carbon and nitrate absorb and transfer light energy (indirect photolysis) to catalyse the degradation of atrazine to form cyanuric acid (Cessna 2008). Corrosive and toxic gases are formed during the incineration process according to the component of the pesticide incinerated. For example, pesticides containing chlorine can produce hydrochloric acid, and nitrogen-containing pesticides can produce nitrogen oxide and nitrogen dioxide during incineration. All the above gases are acidic and corrosive. These toxic exhaust gases are to be treated before letting it out to the environment.

2.3 Bioremediation

Bioremediation refers to the process of detoxifying the contaminated environments using microorganisms, plants, or their enzymes. This includes partial or complete transformation (mineralisation) of the pollutant via biodegradation process. Bioremediation is carried out by adding an enriched microbial culture capable of degrading the pollutant or by stimulating the native xenobiotic degrading bacteria. The major advantages of bioremediation process are that it is environment friendly and cost-effective. Benoit et al. (1998) reported the immobilisation of atrazine by

fungal biomass in soils enriched with lignocellulosic materials where the density of fungal mycelia may be high. Immobilisation of microbial cells on solid porous structures is used for bioremediation of triazine pesticides in water (Yu et al. 2019).

2.3.1 Phytoremediation

Phytoremediation has been suggested as an alternative bioremediation technique to the microbial degradation of pesticide-contaminated sites. Phytoremediation involves the use of vegetation for the in situ treatment of contaminated soil. Though phytoremediation may take longer period for cleaning up the contaminated sites, it is extremely useful for the sites with higher pesticide concentration that will inhibit the microbial growth and activity. Phytoremediation helps in enhancing the organic carbon in soil which helps in microbial growth. Phytoremediation occurs via four mechanisms: (i) direct uptake and accumulation of pesticides and subsequent metabolism in plant tissues are efficient mechanism of pesticide removal, (ii) transpiration of volatile organic hydrocarbons through the leaves, (iii) release of exudates that stimulates microbial activity and biochemical transformations in the soil, and (iv) enhancement of mineralization at the root-soil interface by microorganisms (Schnoor et al. 1995). Phytoremediation can be a cost-effective and eco-friendly way of atrazine degradation. Pesticide-tolerant and nontarget plants can uptake and transform the pesticides to lesser toxic metabolites. Kawahigashi et al. (2006) proposed phytoremediation of atrazine using transgenic rice plants expressing human cytochrome P450 genes CYP1A1, CYP2B6, and CYP2C19. Sanchez et al. (2019) indicated that the atrazine removal from soils was improved by the electric field coupled to phytoremediation.

2.3.2 Biostimulation and Bioaugmentation

Biostimulation is the method of adding appropriate and limiting nutrient amendments to soils to enhance the rapid growth of indigenous bacteria, thereby increasing atrazine degradation rate (Getenga 2003; Qiu et al. 2009). Essential nutrients in limiting quantities usually control the growth of native microbial population. The atrazine removal varies significantly depending on the concentration of atrazine, stimulant type, pH of medium, and inoculation time (Dehghani et al. 2019). Biostimulation will not be effective when the bioavailability of the pesticide is low. Bioaugmentation is proposed for rapid and cost-effective cleaning of atrazine-contaminated sites (Zhao et al. 2019). The addition of layered double hydroxide bionanocomposites (Alekseeva et al. 2011) and carbon nanotubes (Zhang et al. 2015) has been reported to enhance the atrazine biodegradation rate. Bioaugmentation is not that much successful in field trials due to the poor environmental adaptability of the degraders, reduced bioavailability of atrazine, readily available carbon and

nitrogen sources, low utilization of additive substrates, and other complex environmental condition that affects the growth and metabolic activity of the atrazine degraders. The addition of poultry manure increased atrazine removal two-fold as compared to that of control (Gupta and Baummer 1996). Lin et al. (2018) studied the role of earthworm in microbial degradation of atrazine. Earthworms accelerated atrazine degradation by consuming soil humus, neutralizing soil pH, altering bacterial community structure, excreting the intestinal atrazine-degrading bacteria, and enriching indigenous atrazine degraders. Biostimulation and bioaugmentation helps to reduce the atrazine concentrations significantly in heavily contaminated soils.

2.3.3 Enzymatic Bioremediation

Enzymatic bioremediation will be a futuristic approach in resolving the pesticide-contaminated sites especially when the usage of genetically modified or engineered microorganisms is restricted by government regulations. Enzymes help to overcome the most disadvantages pertaining to the use of microbes and plants. Atrazine-degrading enzymes perform well in soil having high nitrogen content which supress the atrazine degradation pathway system in the microbial cells. Enzymes can reach the soil pores which are inaccessible to microbes and will be active in the presence of microbial predators or antagonists. The enzymes are highly selective in degrading the pollutants when the microorganisms prefer the more easily available carbon and nitrogen sources (Rajendran et al. 2018). *Aspergillus* laccase immobilised on biosorbents prepared with peanut shell and wheat straw has a strong potential for the effective removal of pesticides including atrazine and prometryn from water and soil by biosorption coupled with degradation (Chen et al. 2019). Enzymatic bioremediation also suffers from some drawbacks. The free enzymes may be degraded rapidly by the proteases released by the native soil microorganisms. Some enzymes require cofactors which have to be applied along with the enzymes. Further higher purity of the enzymes is much costlier compared to the use of microorganisms. They require optimal environmental conditions for maximum activity. Enzymes may reduce or lose their activity upon pesticide transformation and require repeated applications. Enzyme immobilisation offers long-term stability and can be reused or recovered. Enzymes can be immobilised on natural or synthetic supports through various immobilisation mechanisms. Immobilised enzymes have been reported to have higher stability and activity than the free enzymes.

2.3.4 Factors Affecting Atrazine Biodegradation

Environmental and soil conditions such as temperature, soil pH, structure, type, moisture content, nutrient availability, cation exchange capacity, fertility, organic matter, oxygen, and bioavailability of *s*-triazine pesticide greatly vary and affect the

biodegradation process. Atrazine was found to adsorb to humic acids and clays and to the various interrelated physical and chemical mechanisms of soil (Moreau-Kervévan and Mouvet 1998). Nitrogen compounds have been shown to have negative effect on atrazine degradation by numerous bacterial strains tested in pure cultures and in soil (Alvey and Crowley 1995; Entry et al. 1993; Garces et al. 2007). However, *Agrobacterium radiobacter* J14a (Bichat et al. 1999) and *Arthrobacter* sp. SC-JAK2 (Rajendran et al. 2018, 2019) are not influenced by the simultaneous presence of ammonium, nitrate, and urea in the growth medium. Atrazine-degrading enzymes are inducible in resting cells, if cells are acclimated in media containing growth-limiting nitrogen source, atrazine, or a pathway metabolite. However, their presence in media containing other nitrogen sources did not stimulate the atrazine degradation indicating that these microorganisms prefer the other nitrogen sources for their growth and metabolism. Low atrazine biodegradation is mainly attributed to its low water solubility and migration to soil pores inaccessible to microorganisms. Although addition of surfactants enhances their solubility, they inhibit the microbial activity. Atrazine mineralization rate increases with the increase of water content up to 40% of field capacity. Mineralization was proportional to the organic matter content of the soils and oxygen content. Atrazine mineralization was found to be much slower under denitrifying conditions (Nair and Schnoor 1994). In spite of the presence of significant populations of native atrazine-degrading microorganisms, their ability to significantly degrade the atrazine under complex environmental conditions appears to be limited.

2.4 Nanotechnology in Removal of *S*-Triazine Pesticides

Nanoscale materials are of significant research interest over the past several years because of their improved properties when compared to their bulk form. Nanomaterials including silver, titanium dioxide, and zinc oxide were used as photocatalysts for the heterogeneous degradation of pesticides. Zero-valent metals have been extensively researched for their usage in environmental remediation due to the strong reductive activity. Iron-based nanomaterials have obtained considerable attention in environmental remediation due to their high specific surface area, superparamagnetism, non-toxic and economic characteristics, and abundance. There is a concern on the usage of most nanomaterials intended for environmental application due to their toxicological effects on different biological systems. At present, only iron nanoparticles are considered to be safe for the environmental usage and bioremediation purpose. Some of the nanoparticles developed for *s*-triazine pesticide degradation or removal are presented in Table 2.3.

Table 2.3 Nanomaterials used for *s*-triazine pesticide degradation or removal

S-triazine herbicide	Nanoparticle/nanocomposite	Process	References
Ametryn	Iron – functionalised with 1-butyl-3-methylimidazolidium bromide	Adsorption	Ali et al. (2016)
	Er^{3+}:$Y_3Al_5O_{12}$/Pt-(TiO_2-Ta_2O_5)	Sonocatalysis	Li et al. (2017)
Atrazine	*Penicillium* sp. doped with nano Fe_3O_4 in polyvinyl alcohol-sodium alginate gel beads	Biodegradation	Yu et al. (2018)
	Alginate-stabilised silver nanoparticle	Adsorption	Pal et al. (2015)
	Zinc oxide	Ozonation	Yuan et al. (2017)
	Zero-valent copper	Hydroxyl radical-induced degradation	Hong et al. (2017)
	Pd, PdO, and Ag-Pd on hierarchical carbon structures	Degradation	Vijwani et al. (2018)
Simazine	Au–TiO_2	Sonophotocatalysis	Sathishkumar et al. (2014)
	Diatomite-supported Zero-valent iron	Degradation	Sun et al. (2013)
	Al_2O_3 and Fe_2O_3	Sorption	Addorisio et al. (2011)
Propazine	Titanium dioxide	Photocatalysis	Konstantinou et al. (2001)
Cyanazine	Titanium dioxide	Photocatalysis	Konstantinou et al. (2001)
Prometryne	Titanium dioxide	Photocatalysis	Konstantinou et al. (2001)

2.5 Isolation of *S*-Triazine-Degrading Microorganisms

Several triazine-degrading bacteria were isolated from agricultural and other contaminated sites in various geographical regions. Minimal salt media containing varying concentrations of *s*-triazine herbicides as the sole source of carbon and/or nitrogen were used to enrich and isolate the efficient degraders. Enrichment can be carried out by transferring the initial culture to fresh media containing herbicide every week (up to 5–6 weeks). When the herbicide is first applied, a lag period is observed before degradation proceeds. On subsequent exposures, immediate rapid degradation of atrazine takes place. The addition of carbon source enhances the microbial growth which in turn stimulate the biodegradation process. Nitrogen amendment to the media inhibits the atrazine degradation in many bacterial strains (Entry et al. 1993; Garces et al. 2007). Repeated exposure to atrazine increases the degradation efficiency of the microorganism. After enrichment, potential atrazine-degrading isolates can be identified by clearance zone around the colony on minimal media agar plates containing triazine pesticide. Very limited reports are available on anaerobic degradation of triazines compared to aerobic condition, perhaps due to

the difficulty in working with anaerobic cultures and slow growth of anaerobes. Under anaerobic conditions, soil samples can be enriched using either sulphate (20 mM of sodium sulphate) or nitrate (20 mM of potassium nitrate) as electron acceptors.

2.6 Analysis of *S*-Triazine Herbicide Degradation

In the early 1970s, triazine herbicides were analysed spectrophotometrically in the visible and UV regions and by paper- and thin-layer chromatography. Until the last decade, radiolabeled atrazine was used to study the uptake and detoxification of atrazine followed by quantification with thin-layer chromatography or high-performance liquid chromatography (HPLC). Advanced chromatographic approaches such as GC and HPLC have been developed for the detection of triazine herbicides. Abbas et al. (2015) reviewed the application of gas chromatography and high-performance liquid chromatography for analysis of triazine herbicide residues in various samples. Li et al. (2008) extracted atrazine with dichloromethane from soil and liquid media and analysed with gas chromatography system equipped with a flame ionization detector. Alkali flame detector and electrolytic conductivity detector were used together with a flame photometric detector which is specific for methylthiotriazines and microcoulometric detector specific for chlorotriazines for analysis of specific triazine herbicides. ^{63}Ni electron capture detector was used to analyse halogenated compounds including atrazine.

HPLC is often the method of choice after extraction process. The major advantage of *s*-triazine analysis with HPLC is that it does not require chemical derivatization normally required for gas chromatography analysis. HPLC also provides accurate analysis even in the presence of interfering compounds with GC such as n-alkanes which do not absorb UV light at the wavelength chosen for triazine quantification. Pacáková et al. (1988) separated 18 *s*-triazine derivatives using reversed-phase C18 columns with both UV and amperometric detection by HPLC. UV detection was good for detection of all triazines, while amperometric is useful for hydroxyl derivatives of triazine compounds. Further confirmation can be done with gas chromatography- or liquid chromatography- mass spectrometry in conjunction with thermospray coupling using either a high-resolution or a quadrupole mass spectrometer. There are several studies of HPLC being used as the preferred technique for triazine analysis.

2.7 Conclusion

Farmers have concern over the ban of atrazine. Atrazine ban will have substantial financial impacts on farmer as well as nation economy. An estimated 2 billion dollars and as much as 343 million dollar were estimated to be the revenue loss

annually by corn and sugarcane industries, respectively, in the USA due to elimination of atrazine (US-EPA 2006). Furthermore, there will be much more loss when all the atrazine-dependent crops are included. Postemergence application of other herbicides involves several risk including (i) crop injury as it is applied directly to the emerged crop and weeds, (ii) greater competition between crop and weeds until herbicide application, and (iii) fewer or lack of emergency remedies for weed control if the application of herbicide is missed due to the bad weather or other factors. Physical and chemical methods have not been effective in detoxifying the herbicides under field conditions. Biological methods are the most practical. Research on phytoremediation for *s*-triazine-contaminated soil is limited. Microorganisms have inherent ability to degrade triazine pesticides by utilising them as carbon and nitrogen source. Several reports concluded that excellent atrazine degraders in laboratory media fail to do that in the complex natural environmental conditions that are suboptimal for growth or repress the synthesis of enzymes involved in the degradation pathway. In these cases, enzymatic bioremediation is the excellent solution available now. Since these herbicides are often used in combination with other pesticides, the remediation approaches must able to cope up and degrade or remove these multi-pesticides. The limiting factor in atrazine biodegradation is the lack of efficient atrazine-mineralising microorganisms that can cleave the triazine ring. Much research has to be focussed on biostimulation, bioaugmentaion, and developing recombinant strains to cope up these conditions. With the help of advanced molecular and physiological approaches, such as fluorescent in situ hybridization, denaturing- and temperature-gradient gel electrophoresis and phospholipid fatty acid analysis, and community-level physiological profiling, it is possible to monitor the bioremediation and microbial community development in the atrazine-contaminated soil. The degradative potential of atrazine compromised sites can be established using the primers for the atzABC enzymes.

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Chapter 3
Toxic Effects of Pesticides on Avian Fauna

Anindita Mitra, Soumya Chatterjee, Mainak Sarkar, and Dharmendra Kumar Gupta

Contents

Abstract The best-selling book 'Silent Spring' (1962, by Rachel Carson) quivered the international awareness about the role of pesticides for fatally damaging the avian population, but, after more than five decades of such information and advent of various other functionally similar chemicals, still there are overwhelming recent reports of toxicity of pesticides. Globally, over 5 billion pounds of conventional pesticides are used annually for various purposes; these contaminants may act as silent killer of birds. Several studies suggest that the different populations of birds such as songbird, peregrine falcons, ospreys, and Swainson's hawks are unwitting victims of pesticide contamination.

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Popular organochlorine pesticides like DDT (dichlorodiphenyltrichloroethane) have been replaced by moderately toxic broad-spectrum insecticides, including organophosphates and carbamates and synthetic pyrethroids. Unfortunately, exposures to the organophosphates and carbamates still pose major threats to the different avian species. Having being important component of the ecosystem and their plentitude and sensitivity to direct and indirect effects of environment birds make themselves best indicators of early warning of any environmental problems and threats. Species of different shore, grassland, farmland, and migratory birds are directly exposed to lethal doses of these pesticides or through secondary poisoning.

Both organophosphates and carbamates are anti-cholinesterase chemicals, and intoxication emanates through the inhibition of acetyl cholinesterase, resulting in an accumulation of acetylcholine at synaptic junction following subsequent activation of cholinergic receptors which leads to respiratory damage and eventual death. Avian exposure to these pesticides occurs through dermal contact, inhalation, and predominantly the ingestion of contaminated foods such as seeds or insects. A number of factors intensify the likelihood of exposure of birds to pesticides such as cultivation practices, pest types, crop types, pesticide form, diet, and habitat preferences. The sublethal effects of organophosphates and carbamates on birds are manifolds, including malformed embryos, smaller broods, decreased parental diligence, reduced territorial defence, anorexia and weight loss, subdued immune response, lethargic behaviour, greater susceptibility to predation, interference in thermoregulation, endocrine disruption, and inefficiency to orient in the proper direction for migration. Thus, pesticide intoxication reduces the chance of survival and successful reproduction that ultimately perturb to flourish a healthy bird population. The present review tries to encompass the up-to-date information on the succession of the anthropogenic use of pesticide and their consequences on selected bird populations, emphasizing organophosphates and carbamates in particular.

Keywords Birds · Organophosphate · Carbamate · Lethal effect · Sublethal effect

3.1 Introduction

The human population of the world is predicted to increase approximately 10 billion by 2050 (Saravi and Shokrzadeh 2011). With the greater demand for food, intensification of agriculture becomes the primary objective for researchers and implementers. To produce more in the finite land mass, soil amendments (like use of fertilizers) and application of pesticides (for crop protection, as a whole) are common (Köhler and Triebskorn 2013; Gill and Garg 2014). Pesticides become indispensable in agriculture and are used for different purposes like crop protection, stored grain, floral gardens, and eradication of the pests transmitting infectious diseases in plants. Each year throughout the world, nearly 38 billion USD is spent (12.5 billion USD for the USA alone) on synthetic pesticides (US EPA 2011; Germany 2012).

During the early 1950s, organochlorines, the earliest generations of synthetic pesticides, were used in huge quantities in farms and forests. Organochlorines like dichlorodiphenyltrichloroethane (DDT), cyclodienes, and hexachlorocyclohexanes (HCHs) have the property to remain unchanged in the environment for long time. Due to their lipophilic nature or high octanol-water (Kow) and octanol-air partition (Koa) coefficients, warm-blooded organisms (i.e. birds and mammals) cannot excrete easily through, instead getting accumulated in adipose tissue and undergoing biological magnification (Odabasi and Cetin 2012). The book 'Silent Spring' authored by Rachel Carson (Carson 1962) raised the issue of the environmental risks of use of organochlorines especially DDT. The book elucidated publicly for the first time how indiscriminate application of pesticides and other chemicals is polluting water bodies, impairing birds and animals, and causing health problems in humans (Carson 1962). Although most toxic organochlorines (DDT and several cyclodiene compounds) were restricted and banned in many countries, gradually (since 1980), the second-generation, less persistent pesticides like organophosphates and carbamates and synthetic pyrethroids become the popular agriculture pesticides replacing DDT and cyclodienes. Both organophosphates and carbamates exert their effect to both invertebrates and vertebrates through inhibition of acetylcholinesterase at the cholinergic synapses in the nerve endings (Bishop et al. 1998). Accumulation of the neurotransmitter acetylcholine at nerve terminals and neuromuscular junctions leads to incessant transmission having consequences like seizures, respiratory failure, and, eventually, death (Pope et al. 1995; Marrs 1996; Testai et al. 2010) (Fig. 3.1). In human, organophosphate exposure at sublethal doses for longer duration can lead to cancer, diabetes, and neuronal disorders like Alzheimer's and Parkinson's disease (Amani et al. 2016). As organophosphate and carbamates are easily metabolised and readily excreted from the body, they were once considered safe to nontarget organisms. But unfortunately, a number of reports have shown an alarming decline of birds (namely, sparrow-hawk, mallard, and brown pelicans) in the last five decades due to pesticide toxicity (Mineau 1993; Mineau et al. 1999, 2005; Pain et al. 2004; Mineau and Palmer 2013). The second-generation pesticides have the potential to cause lethal effect in birds as birds are more sensitive to cholinesterase inhibitors than other vertebrates (Table 3.1) (predicted LD_{50} (lethal dose) values in sensitive birds are below 1 mg kg^{-1} body weight, whereas in rat this value is <10 mg kg^{-1} body weight) (Mineau et al. 2001; Health and welfare Canada 1987). The rate of binding of cholinesterase inhibitors like organophosphate and carbamates to acetylcholinesterase is more rapid than other vertebrates (Westlake et al. 1983; Hill 1992) because acetylcholinesterase has higher activity in the brains of bird. In different species of avian fauna brain acetylcholinesterase activity ranges from 7.4 to 19.8 μmol/min/g tissue (Shimshoni et al. 2012). As Mineau (2009) described, the abrupt deactivation of the critical cholinesterase in bird's brain and peripheral nervous system leads to 'short-circuiting' neural connections having a multitude of fatal consequences.

As birds play a central role in ecosystem functioning, healthy avian populations are symbolized for ecological stability because they are highly potential for rapid detection of environmental damages (Wayland et al. 2001; Smits and Fernie 2013).

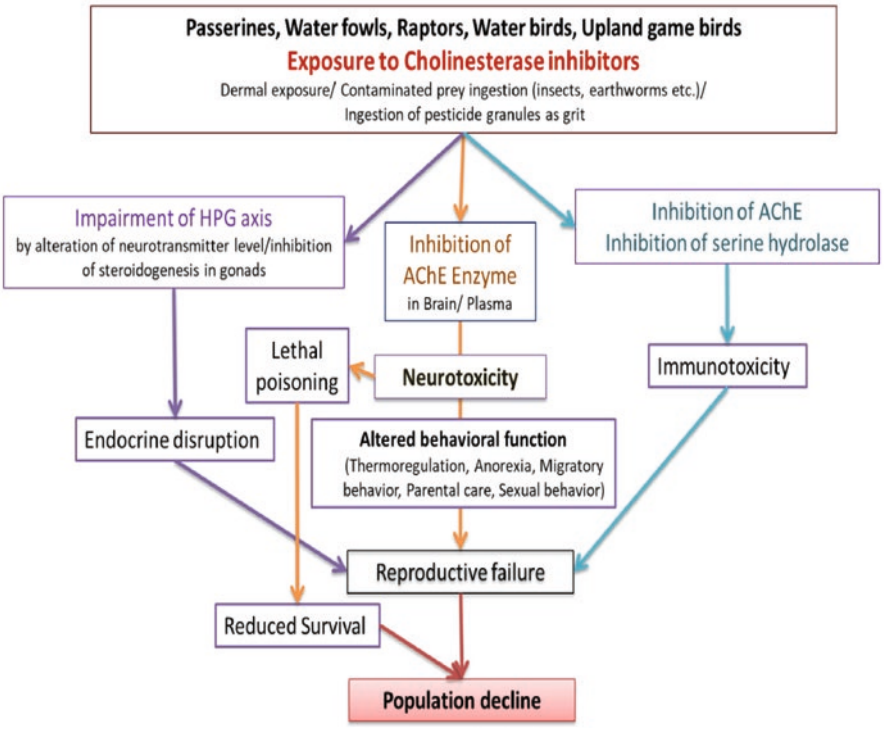

Fig. 3.1 Routes of exposure to cholinesterase inhibitors and their effects on avian fauna
Common routes of exposure of birds to organophosphate and carbamates include inhalation, absorption through the skin, consumption of treated seeds, vegetation with pesticide residues, contaminated insects, granular formulations as grit, food, carrion killed by a pesticide, food intentionally baited with pesticide, and water contaminated with pesticide from runoff or irrigation. Exposure to pesticide resulted in a significant inhibition of brain and plasma acetylcholinesterase (AChE) activity which consequences to death (lethal poisoning) and altered behavioural function. Exposure to such pesticides also leads to inhibition of serine hydrolase activity in immune system and impairment of hypothalamus-pituitary-gonadal axis which results in immunotoxicity and endocrine disruption; all these physiological and behavioural changes reduce the survival and reproductive fitness of the individual and ultimately lead to population decline

Birds accomplish several ecosystem services, like seed dispersal, pollination, controlling pests and bugs (playing a key role as biocontrolling agent), cleaning up carrion, and fertilizing plants, and reclamation of fragmented ecosystems (Sekercioglu et al. 2004; Alanna 2014, Gill and Garg 2014), therefore synchronizing ecosystem function. As they are sensitive and are also prone to contamination due to their food habits (Parker and Goldstein 2000), they are good indicators to assess the quality of the environment and management practices of any ecosystem, agricultural fields, wildlife, water bodies, etc. providing an early warning for environmental problems (Alanna 2014). Researchers stated that declining avian population is a sign of collapsing ecosystem (US FWS 2002; Kendall 2016).

Table 3.1 Acute toxicity of organophosphorus and carbamate pesticides to birds and rat

Pesticide	Class	Species LD_{50} mg Kg^{-1}				
		Mallard duck	Ring-necked pheasant	Red-winged blackbird	European starling	Rat
Aldicarb	Carbamates	3.4	5.34	1.78	4.22	0.8
Carbaryl	Carbamates	>2564	>2000	56	–	600
Carbofuran	Carbamates	0.480	4.15	0.42	–	11
Methiocarb	Carbamates	12.8	270	4.6	13	15–35
Azinphos-methyl	Organophosphate	136	75	8.5	27	13
Phorate	Organophosphate	0.616	7.5	1	7.5	3.7
Temephos	Organophosphate	80–100	31.5	42	>100	2000
Chlorpyrifos	Organophosphate	75.6	8.41	13	5	145
Parathion	Organophosphate	2.2	12.4	2.4	5.6	13
Fenthion	Organophosphate	5.9	17.8	1.8	5.3–17.8	235
Methyl-parathion	Organophosphate	10	8.21	10	7.5	14
Diazinon	Organophosphate	3.54	4.33	2.00	110	250
Malathion	Organophosphate	1485	167	400	>100	1375
Monocrotophos	Organophosphate	4.76	–	1.00	3.30	18
Fenamiphos	Organophosphate	1.68	0.5–1	–	–	8.1

Adopted from Tucker and Haegele (1971), Berg (1982), Walker (1983), Hudson et al. (1984), Schafer et al. (1983), Smith (1987). LD50 is the single oral dose of pesticide in milligrams per kilogram of bodyweight that is required to kill 50 percent of the experimental population

Mortality of birds due to pesticide exposure may occur through different routes, like dermal contact, ingestion of pesticide granules, treated seeds or contaminated water or prey (i.e. secondary poisoning), and poison baits (Fishel 2011; Guerrero et al. 2012). The familiar second-generation pesticides like carbofuran, monocrotophos, diazinon, parathion, and fenthion are also reported to be accountable for avian mortality (Mineau 2002). As for example, mass mortality of Swainson's hawks due to monocrotophos poisoning, in Argentina during 1995–1996, is a well-known phenomenon, after which this organophosphate compound was banned in Argentina (Goldstein et al. 1999). Data suggests that carbofuran (most toxic carbamate pesticide) has been the common cause of poisoning in birds of prey (Novotny et al. 2011; Vlcek and Pohanka 2012). Mineau et al. (2005) reported one of the largest mortality incidents in Canadian prairies with a death of nearly 800 horned larks (*Eremophila alpestris*) and 2000 lapland longspurs (*Calcarius lapponicus*) happened due to application of carbofuran granules in Canadian agriculture fields. Similarly, spraying of carbofuran for controlling grasshopper led to disappearance of burrowing owls in Canada (Fox et al. 1989). In 2011, DWHC reported remarkably high (830 mg kg^{-1} body weight) aldicarb in the stomach of the carcasses of male crow, male lesser-black-backed gull, and juvenile male and female buzzards.

According to a report of BLI, around 50 types of pesticides are involved for killing of different species of birds like songbirds, raptors, game birds, seabirds, and shorebirds (Birdlife International 2004; http://datazone.birdlife.

org/sowb/sowbpubs#SOWB2004). Studies support the activity of cholinesterase inhibitors inducing increased death of predatory and granivorous birds (such as Neotropical migrants, raptors, passerine, and crane) (Eisler 1985; Agriculture Canada 1993; Mineau 1993, 2005; Mineau et al. 1999; Pain et al. 2004; Mineau and Palmer 2013). Nonetheless, the study of pesticide-induced death of birds is difficult for the authors due to elusive nature of birds, as birds may be exposed and then migrate too far from the exposed areas and may die elsewhere or are consumed by scavengers (Hussain et al. 2011; Mitra et al. 2011).

Most current problems of avian acute toxicity due to pesticides stem from the illegal use of the banned pesticide (Mineau and Whiteside 2006) in many countries. The programme organized by FAO (The United Nations Food and Agriculture Organization) made aware the developing countries in the Middle East as well as Africa about the risks associated with outdated pesticide stocks and how they can be and what they can do about them; additionally, the Prevention and Disposal of Obsolete Pesticides programme organized by FAO also supports the states in discharging the stockpiles and hindering the reaccretion of banned pesticides (FAO 2013). As reported by Mansour (2009), most of the African countries have effectively or partly eradicated enormous quantities of persistent pesticides; still, Ethiopia authorizes one of the eminent reserves of banned pesticides (according to a report of FAO, 2013). In Yemen, up to 70% of pesticides are illegally imported for application on fruit and vegetable crops, specifically the qat tree (*Catha edulis*) (UN Office for the Coordination of Humanitarian Affairs 2007). Indeed, the highest amount of pesticides is sprayed in qat cropping areas in comparison to other vegetable crops promoting birds toward higher exposure and intoxication. This review aims to comprehend the up-to-date information on the succession of the anthropogenic use of pesticides and likelihood of the exposure to the avian fauna and their consequences on selected bird populations, emphasizing organophosphate and carbamates in particular.

3.2 Likelihood of Exposure

Different components like form of cultivation and nature of crop, types of pest, nature of pesticide and their forms, and food habits of birds are responsible for probable exposure pathways to pesticides. Probability of exposure to pesticides also depends on bird's ecology related to diet, foraging activities, habitat preferences, and migration behaviour (BLI 2013). Rather than the diet preferences or daily intake rate, foraging location is more important to influence exposure of birds to pesticides (Corson et al. 1998). Birds that search for food near agricultural areas are at higher risk for pesticide exposure rather than the birds that rummage more intermittently in grassland areas (usually, no insecticide is applied in that area) (BLI 2014).

3.2.1 Cultivation Practices May Influence Likelihood of Exposure

Probability of exposure to pesticides in birds is directly linked with the agricultural practices, like timing of pest control in a particular area for a seasonal crop (Narváez et al. 2016; Santos et al. 2016). A study by Osten et al. (2005) with black-bellied whistling duck (*Dendrocygna autumnalis*) reported the direct correlation during the period between pesticide application for specific agronomic purposes and significant inhibition of ChE activity due to exposure to anticholinesterase agents (organophosphate and carbamates). Further, secondary poisoning (like mortality of raptors due to consumption of contaminated songbird) is also evident due to pesticide application (Mineau et al. 1999). Therefore, coordinating time with pesticide applications and bird's annual cycle is necessary to avoid secondary poisoning. Though most of the second-generation pesticides are biodegradable, some of them may persist for a few months in the environment, water-logged fields, etc. making them available for exposure to birds (Mineau et al. 1999). Pesticide exposure rate to birds also depends upon its applications in croplands in different seasons. As for example, in Switzerland granular carbofuran was applied for seed protection in every spring which was linked to the declining of raptor population in spring (Dietrich et al. 1995). Similar findings have been reported by Mineau et al. (2005) in North American canola fields, where insecticides were applied from mid-May to early-June when migratory songbirds and geese were residing in the canola fields resulting in population reversal in those bird species. In some cropland, exposure risk reduces during growth period of the crops, because some birds prefer to spend time during seedling period of the crop rather than budding stages (Corson et al. 1998).

3.2.2 Types of Pest That Increase Risks of Pesticide Intoxication to Birds

Exposure of pesticides to birds depends upon the insect species they feed on, as pesticides are linked to pest control; therefore, birds feeding on insect pests of agriculture fields are more likely to be exposed to chemicals. Some insect species constitute a large fraction of a bird's aliment, which may be the target of particular pesticides. Rapid re-emergence of a pest or relative abundance in a crop field also prompts heavy pesticide applications (Cutler 2012; Qu et al. 2017). Pest resurgence may occur due to the application of less degradable and wide-ranging pesticides that terminate the native predators to a particular pest, or due to eradication of a primary pest that helps to flourish the secondary pests to emerge and become the major pest (Dhaliwal et al. 2006; Gill and Garg 2014). Pest outbreaks allure avian species and thereby unravel the strong possibility of vulnerability of birds to pesticides. Reports revealed the reappearance of bed bug (*Cimex lectularius*) (Davies et al. 2012), cotton bollworm (*Helicoverpa armigera*) (Mironidis et al.

2013), and brown plant hopper (*Nilaparvata lugens*) (Wang et al. 2010) population due to insecticide/pesticide resistance. To control locusts and grasshoppers, pesticides are sprayed on a large scale often in the habitats outside of the croplands; therefore, avian fauna that rummages ahead of cultivated land may also be prone to the toxicant (Dong et al. 2016). As for example, North American Grasshopper hawks (*Buteo swainsoni*) that prefer insects of Acrididae family (locusts, grasshoppers) as their food are more vulnerable to poisoning because of targeting pest outbreaks in agricultural crops (Mineau et al. 1999). Grasshopper control in Argentina by organophosphate and monocrotophos exterminated nearly 5000 grasshopper hawks during the arid summer of 1995–1996 (Goldstein et al. 1999). Pesticide application targeting pest outbreaks poses more risk to bird species than normal preventive use.

3.2.3 Crop Species Raising the Possibilities of Exposure

Cultivation of some specific crop species may hike the liabilities of pesticide exposure to birds (UNEP/CMS/SCC18/Inf.10.9.1, 2014). Pesticides of particular forms (granular/liquid form) and frequent or higher quantities of applications related to cultivation practices that are required for protection of some crops are more vulnerable for birds. Further, some crop types attract birds with higher foraging areas, food source, or other resources. Rice is one of the best examples that has the highest cumulative risk to birds than other crops. Parsons et al. (2010) reported that in the USA, pesticide treatments for rice cultivation cause death of water birds. Again, paddy field provides attractive winter sojourn and migratory halt sites for shorebirds, posing a high risk of exposure to organophosphate and carbamates. Eventually, the pesticide contamination probability to birds is increasing worldwide due to decrease in natural wetlands and ecosystems (Strum et al. 2008).

3.2.4 Formulation of Pesticides Increasing the Risk of Exposure

Particulate Forms

Granular formulations (grit types) of pesticides are more farmers' friendly (than liquid concentrates or powders) as they are easy to apply, highly concentrated, and target specific and have higher retention capacity in the fields (Best 1995). Consumption of natural grit is common to many bird species (especially for granivores and insectivores), as grit assists in grinding the hard foods such as seeds and hard animal parts in gizzard. Granivorous birds like sparrows, finches, and parrots searching for pebbles may consume granular pesticides primarily through incorporation along with

food (Best 1995). These birds are unable to distinguish granular form of pesticide because the carrier medium of pesticide granules is sand (silica) which is the ideal natural grit material (Mineau 2009). Therefore, particulate forms of pesticide may enter into the body of avian fauna either through intentional or unintentional ingestion and/or absorption through exposed area (Best and Fischer 1992). For example, waterfowl are exposed to granular insecticides when sieving for crop residues in pond and drenched soils or intake of grit (Best and Fischer 1992). Parsons et al. (2010) reported the pesticide contamination in water birds in some submerged crop (corn, wheat, and rice) fields in the USA and Canada. Granular forms of the pesticide differ in size, colour, shape, surface texture, carrier material composition, pesticide load per granule, and activity period in the field after application (Gionfriddo and Best 1996; Stafford et al. 1996). Besides primary poisoning, second-generation granular form of pesticides (such as carbofuran) causes secondary poisoning, for example, in raptors, consuming contaminated birds or other wild animals or been contaminated to such granules led to regional population declines (Sánchez-Bayo et al. 2013).

Seeds Coated with Insecticide

Seed treatment with organophosphate and carbamates is very common in developing countries but having been replaced by neonicotinoids (such as imidacloprid) and is no longer approved in some developed countries. Conventional seed treatment by applying pesticides for crop protection was widely used in the UK during the year between 1992 and 2002 (Garthwaite et al. 2003; Prosser and Hart 2005). In Spain, among 18 pesticides permitted for coating of seeds of cereals (MAGRAMA 2013), thiram and maneb are used as fungicides (among the 14), and the rest four are as insecticides. In India, carbofuran-treated seeds have been widely applied, and as a consequence, a number of birds are regularly exposed, though not being reported till date (Venkataramanan and Sreekumar 2012). These pesticide-coated seeds may serve as food source for granivorous birds, which may be fatal to flocks by wilful consumption and subsequent death of carnivorous birds for consumption of contaminated primary population (secondary poisoning) (Fletcher et al. 1996; Almeida et al. 2010). The incidence of avian poisoning due to seed treatment has been reported by Pain et al. (2004) where 15 sarus cranes (*Grus antigon*) and 3 common cranes (*Grus grus*) were intoxicated by monocrotophos at Bharatpur reserve forest in India. Intoxication from treated seeds depends on various factors like sowing area, lethality of pesticide and its concentration on the seed, density of the disclosed seed, and accessibility of alternative food materials (UNEP/CMS/SCC18/Inf.10.9.1, 2014). Again, the risk of exposure to the treated seeds will depend on how likely the birds are to consume them. Birds can avoid the toxic seeds due to the appearance and repugnance of the food or due to feeling of distress due to sublethal toxic effect through a mechanism of conditioned antipathy (Lopez-Antia et al. 2014).

Liquid Formulations and Flowable Pesticide

Liquid formulation is generally mixed with water, but sometimes, crop oil, diesel fuel, kerosene, or some other light oil may be used as a medium. Liquid formulation of pesticide usually contains the active ingredient, the carrier, and one or more other ingredients and may combine the features of emulsifiable concentrate and wettable powders. Although liquid formulations are easier to apply, they are efficiently absorbed through skin of human and other animals. Liquid pesticides lessen the risk of exposure to birds in comparison to the granular application because granular formulations are more attractive to birds (Mineau and Whiteside 2006). However, liquid form of pesticides can contaminate offsite soil, aquatic ecosystem terrestrial flora, and insects from drizzling droplet overflow and erosion after application, and some pesticides may disperse into the air, either becoming airborne during application or volatilizing from treated surfaces (Mackay et al. 2014). The most likely routes of exposure of flowable pesticides to birds are through the ingestion of residues in food items (contaminated vegetables/prey) and in drinking water (on-field puddles or other sources) or through inhalation, dermal contact, and preening (Moore et al. 2014). The risk of flowable pesticide will be greater to birds foraging on-field than off-field birds as greater exposure occurs in treated field. A study reported that mortality rates per day in the flocks of Pennsylvania mourning dove (*Zenaida macroura*) and American robin (*Turdus migratorius*) were significantly higher in the methomyl, oxamyl (carbamate and dimethoate organophosphate), and sprayed apple orchards than in non-treated orchards, and exposure of doves and robins to these insecticides in a frequent rate significantly lowered the reproductive fitness of these species (Fluetsch and Sparling 1994).

3.3 Lethal Effect of Organophosphate/Carbamate Exposure on Birds

The birds are subjected to be at risk of lethal or sublethal doses of any broad-spectrum pesticide like organophosphate and carbamates, if the time of foraging in the adjacent area coincides with the time of application, or shortly after that, or come in contiguity with the contaminated prey. According to the report of the United States (US) Department of Interior's National Wildlife Health Centre, organophosphate and carbamates are responsible for nearly about 50 percent of the registered cases of lethal poisoning of birds (Madison 1993) that occur due to ingestion of contaminated seeds or through secondary poisoning with the high concentration of insecticide (Prosser and Hart 2005). During the year 1980–2000, about 335 individual mortality cases of approximately 9000 birds in the USA happened due to organophosphate intoxication (Fleischli et al. 2004). The worst organophosphate monocrotophos has been reported to cause over 100,000 deaths of avifauna throughout the world (Hooper 2002). Spraying operation of diazinon to backyard,

playground, and grassland in the USA has reported to kill thousands of birds (Tattersall 1991). Waterfowls like ducks and geese are most sensitive to diazinon (LD_{50} value in Table 3.1), and carbofuran alone is responsible for massive mortality of birds in California followed by diazinon (US EPA 1998).

3.4 Sublethal Effect of Organophosphate/Carbamate Exposure on Birds

The effects of organophosphate and carbamate pesticides are multitude. Sublethal effects of cholinesterase inhibitors include production of smaller broods, changes in mobility, feeding and migratory behaviour, endocrine disruption, immunomodulation, and interference in reproduction (Pinkas et al. 2015). Thus, pesticide intoxication reduces the chance of survival and successful reproduction, ultimately perturbing to develop a healthy bird population (Pinkas et al. 2015; Eng et al. 2017). However, scanty information is available on the probable consequences of long-term exposure to organophosphate/carbamates at sublethal level on avian species.

3.4.1 Biochemical Biomarkers for Anticholinesterase Contaminants

Potential biomarkers used to monitor anticholinesterase exposure in birds include determination of cholinesterase (Santos et al. 2016) and carboxylesterase activity (Barr and Needham 2002), assessment of oxidative stress (Henry et al. 2015), and lactate dehydrogenase activity (Barata et al. 2010). However, application of suitable biochemical biomarkers depends on prior knowledge of species-specific and age-related reference and threshold values to identify stress-mediated responses from natural background variation (Domingues et al. 2015; Santos et al. 2019). A dose-dependent decrease in liver carboxylesterase activity was found following exposure to malathion, parathion, and trichlorfon in the range 0.125–2 mM for 48 hrs in Japanese quail (Abass 2014). Similar findings were observed in chlorpyrifos-treated Japanese quail by Narvaez and coworkers (2016). In a recent study, Ćupić Miladinović and coworkers (2018) observed that following chlorpyrifos (CPF) exposure, there was an increased accumulation of ROS in brain cells of Japanese quail (*Coturnix japonica*), supporting generation of oxidative stress. They have also reported the alteration of several oxidative stress-related parameters such as increased concentration of malondialdehyde (MDA), glutathione (GSH), nitrite (NO_2^-), and hydrogen peroxide (H_2O_2)] and increased activity of antioxidative enzymes like superoxide dismutase (SOD) and myeloperoxidase (MPO).

3.4.2 *Neurotoxic Effect*

As discussed, organophosphates and carbamates are well known for inducing cholinergic overstimulation (neurotoxic effect) by inhibiting acetylcholinesterase activity which leads to collection of acetylcholine at nerve terminals (Fig. 3.2) and neuromuscular juncture overstimulating nicotinic and muscarinic receptors (Walker and Thompson 1991; Walker 2003; Testai et al. 2010; Ivanović et al. 2016). Brain acetylcholinesterase is a potential biomarker for monitoring the degree of exposure to anticholinesterase agents and thus assessing the effect of these pesticides on bird population (Busby et al. 1983; Smith et al. 1995; Timchalk 2010; Villar et al. 2010). The anticholinesterase-degrading enzymes were found in reduced level in birds which may be responsible for higher sensitivity of birds to anticholinesterase pesticides than other vertebrates (Parker and Goldstein 2000). However, plasma acetylcholinesterase activity may also be considered as a promising indicator of the central nervous cholinergic status (Oropesa et al. 2013). Studies suggest that in brain of birds, greater than 50% inhibition of cholinesterase activity may lead to death (Ludke et al. 1975; Mineau et al. 2001; Mohammad et al. 2008). In contrast to organophosphate poisoning, post-mortem reactivation of cholinesterase may provide false-negative results in cases of carbamate poisoning (Smith et al. 1995).

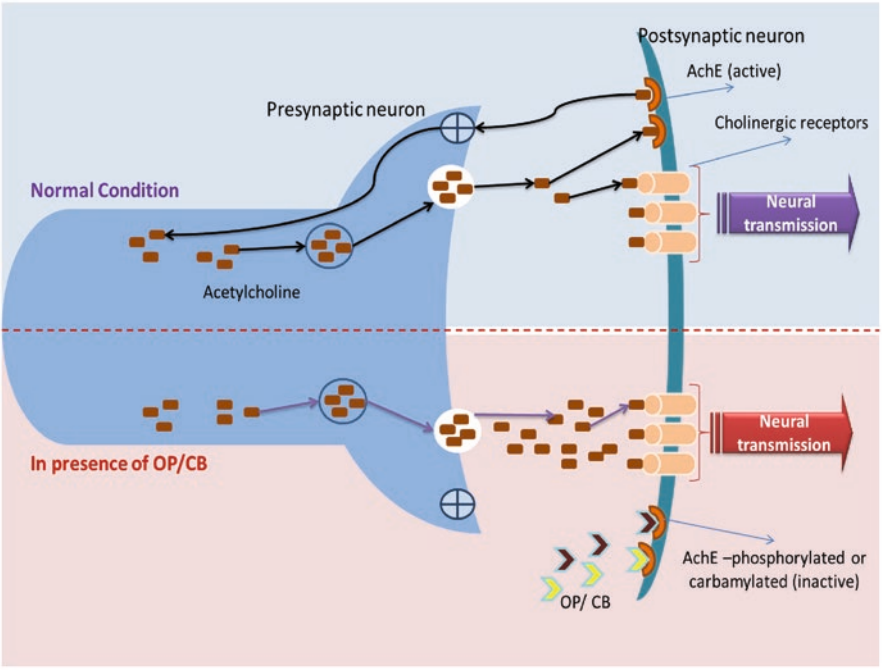

Fig. 3.2 Mechanism of inhibition of acetylcholinesterase (AChE) by organophosphates and carbamates

The reactivation of carbamylated cholinesterase is dependent on the duration and the temperature at which the carcass remained in the field and would therefore help in masking carbamate poisoning (Smith et al. 1995). Secondary symptoms may occur in some organophosphate poisoning called organophosphate-induced delayed neuropathy (OPIDN) in which the target enzyme is brain neuropathy target esterase (NTE), manifested by limb immobility of exposed individual (Lotti and Moretto 2005). OPIDN is symbolized by the demyelination of neurons and paralysis which can be noticed after 20–25 days following single or recurrent exposure(s) (Grue et al. 1997). Neurotoxic effect of organophosphate may also be mediated through oxidative damage by producing reactive nitrogen species [such as nitric oxide (NO•) and nitrogen dioxide (NO_2)]. Excess (NO•) acts as a neurotoxin-promoting neurodegenerative disorders (Di Meo et al. 2016).

Impairment of mitochondrial function plays an important role in the progress of many neurodegenerative disorders (like Parkinson's disease, Alzheimer's disease), which is related to the inhibition of complex I (CI) or ubiquinone oxidoreductase, member of the oxidative phosphorylation system located at mitochondrial inner membrane (Kulic et al. 2011). CI inhibitory effects were found in rats exposed to monocrotophos (Masoud et al. 2009) and dichlorvos (Binukumar et al. 2012), which include acute cholinergic conflict succeeded by possible intermediate syndrome and finally OPIDN. A study reported that hens treated with chlorpyrifos showed sign of delayed neuropathy like ataxia and locomotor disturbances with concomitant inhibition of NTE, mitochondrial CI, and decreased ATP (adenosine triphosphate) production, thereby supporting organophosphate-induced mitochondrial dysfunction in birds (Salama et al. 2014) which may be indirectly responsible for the changes in behaviour due to neurological complications.

Exposure to pesticide at sublethal dose emanated to a significant hindrance in brain cholinesterase activity in reproductively mature adults that can be correlated to an extent behavioural alteration such as limited mobility and interrupted incubating ability; all these changes ultimately lead to considerable lower production of fledged young (Busby et al. 1990). Studies have demonstrated that cholinesterase-inhibiting insecticides exclusively affect almost all physiological and behavioural functions (Greaves and Letcher 2017). Behavioural changes in response to toxic chemicals provide an insight to the population and ecological repercussion in remote future (Raley-Susman 2014). Organophosphate may induce behavioural alteration in avifauna by interfering thermoregulation, food consumption, sexual behaviour, clutch size, embryonic development, mobility, seasonal behaviour, territorial behaviour, and parental care (Grue et al. 1991, 1997). Such switch-over in physiological and behavioural pattern has the potential to reduce the survival and reproductive fitness of individuals, which ultimately affects the population up to local extinction of several bird species (Grue et al. 1997). A perceptible change in a population of white-throated sparrows (*Zonotrichia albicollis*) in Canada took place after forestry spraying operations with fenitrothion (an OP) (Busby et al. 1990). Lethargic behaviour of birds due to sublethal exposure of organophosphate and carbamates can increase the susceptibility for predation of house sparrows (*Passer domesticus*) and bobwhite quails (*Colinus virginianus*) (Hunt et al. 1992; Hawkes

et al. 1996). Red-winged blackbirds (*Agelaius phoeniceus*) exposed to sublethal dose of organophosphate for longer duration was found to affect the feeding behaviour of the species (Nicolaus and Lee 1999). As sublethal exposure of these pesticides is often associated with anorexia, a common consequence of exposure is reduction in the body weight (Grue et al. 1991; Maitra and Mitra 2008; Moye and Pritsos 2010). A single dose of dicrotophos leads to 55–77% inhibition of brain acetylcholinesterase in European Starlings that can be correlated with weight loss (Grue and Shipley 1984). According to Kuenzel (1994), pesticide-induced lesion in lateral hypothalamus leads to food avoidance causing significant body weight reduction in birds. Pigeons exposed experimentally to sublethal dose of chlorpyrifos and aldicarb showed abnormal flight and improper navigation (Moye and Pritsos 2010). Mc Carty et al. (2009) reported that buff-breasted sandpiper (*Tryngites subruficollis*), a species of migratory shorebird that sojourn in spring at the agricultural fields near Rainwater Basin area of Nebraska, is subjected to extensive exposure to pesticide that led the bird to debilitate social and courtship behaviour (Mc Carty et al. 2009).

Organophosphates and carbamates affect thermoregulatory ability in birds causing inability to withstand in cold seasons (Martin and Solomon 1991). Acute exposure of organophosphate at sublethal level showed pronounced but transient hypothermia (Grue et al. 1991). Anticholinesterase-directed hypothermia in birds is often coupled with more than 50% inhibition in brain acetylcholinesterase activity (Clement 1991). The higher mortality rate in American kestrels (*Falco sparverius*) exposed to cold temperature was due to poor thermoregulatory ability (Rattner and Franson 1983).

3.4.3 Effects on Endocrine System and Reproduction

Organophosphates and carbamates are among the endocrine-disrupting chemicals (EDCs) that can cause altered patterns of behaviour by mimicking the action of hormones. Although effects of exposure to EDCs vary from species to species, the bird species at the top of the food chain are particularly vulnerable (Carere et al. 2010). EDCs can interrupt redox homeostasis causing oxidative stress and imbalance between pro-oxidants and antioxidants. Redox balance shifted toward more oxidant condition instigates oxidative damage, anticipating several degenerative pathologies (Abdollahi et al. 2004).

Damstra et al. (2002) suggested that some unique characteristics of birds make them more vulnerable to potential endocrine-disrupting chemicals including consumption of large amount of food and higher metabolic rates, periods of starvation that mobilize lipid reserves, hormone-dependent behaviours, developmental scheme, and regulation of sexual differentiation. Physiological and metabolic processes of a bird can be considerably affected by very delicate changes in the balance of the endocrine system (Damstra et al. 2002; Fernie et al. 2015).

Although organochlorines (prochloraz, endosulfan, dieldrin) are well known for their estrogenic properties, some of organophosphates and carbamates (toclofos-methyl, chlorpyrifos, quinalphos) are also reported to have estrogenic potential (Andersen et al. 2002; Kitamura et al. 2010). Differentiation of avian reproductive system is estrogen dependent; therefore, differentiation of the accessory male and female genital ducts was found to be altered by estrogen exposure in a dose-dependent fashion (Fry 1995). Chatterjee et al. (1992) reported estrogen-like action of quinalphos exposure of which induces vaginal cornification increased uterine wet weight in rat as established by uterotrophic assay. Gonadal impairment may occur due to alteration in steroidogenesis in testis or ovaries of the birds treated with anticholinesterase agents (Ray et al. 1987). Sublethal exposure of organophosphates (methyl parathion/phosphamidon/quinalphos) to wild female of white-throated munia (*Lonchura malabarica*) showed significant reduction in the activity of two important steroidogenic enzymes of growing follicle, namely, Δ53β-hydroxysteroid dehydrogenase (3βHSD) and 17β-hydroxysteroid dehydrogenase (17βHSD) in a dose-dependent manner (Mitra 2006). These two steroidogenic enzymes (3βHSD and 17βHSD) are the key player in the production of oestrogen and progesterone, respectively (Civinini et al. 2001). Degenerative changes at histological level in ovary of the treated birds include reduced thickness of membrane granulosa layer, vacuolation, and exfoliation of granulosa cells of mature follicles (Mitra 2006). Arrested developmental process and degeneration of spermatogenic cells are observed in domestic and semi-domestic birds after exposure to organophosphates (Mitra et al. 2011). Increased number of degenerated germ cells in the seminiferous tubules was found in the testis of adult male white-throated munia (*L. malabarica*), after exposure to sublethal dose of methyl parathion (Maitra and Sarkar 1996). Male rose ringed parakeets (*Psittacula krameri*) when ingested to graded doses of methyl parathion showed subsided testicular function which may be caused by an upset circulating context of LH and testosterone (Maitra and Mitra 2008). Thus, sublethal exposures of xenobiotics adversely effect on reproduction of avian fauna that may not be related to mortality but through a direct effect on population level. Alternative pathway of organophosphate-induced reproductive impairment in different vertebrates may occur by modifying neurotransmitter levels and thus debilitating hypothalamic and/or pituitary monitoring on reproduction (Muller et al. 1977). Possibilities also exist by suppressing GnRH release, which may act directly by modifying gonadotropin synthesis and secretion or indirectly by changing the pituitary cell sensitiveness to GnRH through the mediation of gonadal steroids resulting from adjustment of FSH and LH level by feedback mechanism (Stoker et al. 1993). According to Rattner et al. (1984), organophosphorus insecticides depreciate reproductive function possibly by modulating secretion of luteinizing hormone and progesterone. Rattner et al. (1982) also reported that significant decline in plasma titre of LH, progesterone, and corticosterone was noted in female bobwhite quail following the short-term exposure of parathion.

3.4.4 Effects on Immune System

The immune system is predisposed to any external insults including xenobiotics (Blanco 2011). Impairment of immune system of vertebrates by organophosphate/carbamates has been evidenced by a number of research works in the past decades (Wong et al. 1992; Barnett and Rodgers 1994; Vial et al. 1996; Zhuang et al. 2015). Decreased humoral and cell-mediated response and nonspecific immunity, along with increment in hypersensitivity and autoimmunity, are some immunotoxic effects induced by organophosphate agents (Shahzad et al. 2015). Normal functioning of immune system is impeded through anticholinergic as well as non-cholinergic pathways by organophosphate (Barnett and Rodgers 1994; Vial et al. 1996). Anticholinesterase-induced oxidative stress and immunomodulation are well established in mammalian models (Cabello et al. 2001; Galloway and Handy 2003; Abdollahi et al. 2004; Polláková et al. 2012; Watanabe et al. 2013). Unfortunately, very little information is available about organophosphate-/carbamate-induced immunotoxicity in non-mammalian models like birds. Cupic Miladinovic et al. (2018) have suggested that chloropyriphos-induced oxidative stress in Japanese quail may be responsible for inflammatory responses. Immunosuppressive effects like reduced lymphocyte proliferation and reduced functional status of phagocytic cells have been found in carbaryl-treated chicken (Singh et al. 2007). Young chicks when exposed to sublethal dose of chloropyriphos and methidathion resulted in reduction of total count of WBC, neutrophils, and lymphocyte (Obaineh and Matthew 2009). In the study of Shahzad et al. (2015), there was increased proliferation of interfollicular connective tissue, cytoplasmic vacuolation, oedema, and appearance of pyknotic and fragmented nuclei (marker for degeneration that depleted the frequency of lymphoid follicles in bursa of Fabricius in chlorpyrifos-treated chicks at sublethal dose). Similar degenerative pathologies were also found in spleen and thymus of the treated chickens.

3.5 Pesticides and Birds

In northern Europe and North America, many grassland or farmland bird species are known to undergone population declines in the past five decades (Mineau and Whiteside 2013). Reports from various survey analyses indicated that grassland birds of North America as a group are declining faster than birds from other biomes (Dunn et al. 2000; Sauer et al. 2000; Bird Life International 2013). During 1971–1975 after introduction of organophosphate in the UK, a series of incidents involving mortality of birds have followed after application of herbicides and insecticides for agricultural intensification in UK, primarily via indirect, food-mediated effects (Campbell and Cooke 1997; Potts 1997). Following consumption of coated seeds with carbofenothion resulted in mass mortality of greylag geese (*Anser anser*) and pink-footed geese (*Anser brachyrhynchus*) in the UK, assumed to be around 1%

of the world population of this species (Greig-Smith 1994). This upshot in the UK led to restrictions of the use of carbofenothion and replacement by chlorfenvinphos; however, chlorfenvinphos poses a greater risk to pigeon than to geese. The increased mortality of pigeon leads to replacement of chlorfenvinphos by fonofos during the middle of 1980s (Greig-Smith 1994).

A study to discover out the prime factors for grassland bird declines in Europe and North America based on the data for 23-year period (from 1980 to 2003) was organized by the toxicologist Pierre Mineau and others (Mineau and Whiteside 2013). The study analyses the five potential sources of grassland bird decrement besides fatal pesticide menaces. These are change in agricultural farming such as hay or alfalfa production, farming intensity, or the percentage of actively cropped agricultural pasture, the use of herbicides and insecticide, and change in permanent pasture and rangeland. This study recognizes that the foremost cause of the extensive decline in grassland bird numbers in the USA is acutely toxic pesticides and focuses on the degree to which lethal pesticides, like organophosphates and carbamates, are accountable for the decrement in grassland bird populations. This finding challenges the most likely assumption that change in crop pasture, the chief factor for habitat loss, was the primary cause of those population declines (Mineau and Whiteside 2013).

Pesticides are recognized as one of the cause of the frequent declines of species of Neotropical migrants. From a report of American Bird Conservancy (2009), among the 341 species of Neotropical migrants (includes plovers, terns, hawks, cranes, warblers, and sparrows), 127 bird species were known to decline. Sixty species were in severe decline mode (population decrease of 45% or more), of which 29 were different species of songbird. Due to destruction of natural swampland and grasslands (Knopf 1994; Skagen 2006), shorebirds are enforced to inhabit alternative places like paddy fields and turf grass farms as migratory stopover during their annual journey between breeding and non-breeding territory (Twedt et al. 1998; Corder 2005; Blanco et al. 2006; Robbins 2007). Although application of highly toxic organophosphate and carbamates is regulated in many countries, less toxic organophosphate and carbamate compounds are still recommended to be used in agriculture for controlling pest of the crops including rice and turf grass in various countries of North and South America (Merchant 2005; Blanco et al. 2006; Way and Cockrell 2007). Poisoning of migratory birds in South America has been documented to potential exposure to cholinesterase arresters while utilizing resources in agricultural habitats (Goldstein et al. 1999). Feet of dead birds may be used to monitor the short- and long-term external (dermal) exposure to pesticides beside the traditional method using chemical analysis of pesticide present in the gastrointestinal tract, liver, eggs, and muscle (Alharbi et al. 2016). In addition to mortality (Pain et al. 2004; Wobeser et al. 2004; Renfrew et al. 2006), sublethal exposure to organophosphate and carbamates can elicit a number of behavioural changes such as loss of migratory orientation and slower flight speed due to obstruction in physiological process (Vyas et al. 1995; Grue et al. 1997; Brasel et al. 2005).

Migrants belonging to Nearctic-Neotropical shorebird can be categorized into upland and wetland shorebirds depending on their habitat requirements. Upland

shorebirds like American golden-plover (*Pluvialis dominica*), upland sandpiper (*Bartramia longicauda*), and buff-breasted sandpiper (*Tryngites subruficollis*) prefer dry habitats having low vegetation (Myers and Myers 1979; Isacch and Martínez 2003). The aforesaid species regularly utilize crop pasture migratory sojourn (Strum et al. 2008) and devour a variety of agricultural pests, whose emergence time coincide with the migration period of birds and, thereby, may come into direct contact with organophosphate and carbamates (Houston and Bowen 2001; Nagoshi and Meagher 2004; Isacch et al. 2005). On the other hand, wetland species, such as least sandpiper (*Calidris minutilla*), pectoral sandpiper (*C. melanotos*), and white-rumped sandpiper (*C. fuscicollis*), prefer habitats with standing water and intermittently visit rice fields and other agricultural areas where organophosphate and carbamates are widely used (Hands et al. 1991; Skagen and Knopf 1993; Twedt et al. 1998; Skagen et al. 2005; Blanco et al. 2006). Carcasses of few shorebirds have been found in rice fields shortly after carbofuran (a potent anticholinesterase) application, as reported from a number of field survey (Flickinger et al. 1980, 1986; Littrell 1998).

3.6 Conclusion

Application of synthetic pesticides was initially aimed to intensify agricultural productivity and food availability; however, their negative effects have overweighed their welfares. From the past decades, prevalence of pesticide poisoning of birds and the hazardous effect due to direct and indirect poisoning of synthetic pesticides cannot be overlooked; second-generation insecticides, organophosphates, and carbamates are used in routine agricultural practices, and their effects on avian fauna are varied depending on the plausibility of exposure and the degree of toxic level exposure of the pesticide. However, the likelihood of exposure and related lethal and sublethal effects of the pesticides are formidable to study at the field level. Conversely, it can be stated that the broad-spectrum nature of organophosphates and carbamates makes birds more liable to of exposure, when they are present in the neighbouring areas at the time of applying pesticide, or immediately after that, or getting contact with a pesticide contaminated prey. Migratory birds are more prone to sublethal effects of pesticides having cholinesterase inhibitors that can have significant effects on migratory behaviour of the birds.

The above discussion tries to encompass the severe consequences of indiscriminate pesticide application on avian population. To understand the range to which birds are exposed to pesticides, the foremost requirement is to find out the factors influencing exposure and to figure out preventive measures that might be applied to minimize the exposure. Best management practices integrating control in agricultural practices, use of pest resistant cultivars of plants, and rational use of synthetic pesticide could reduce hazards of pesticide application. Further, advanced approaches in biotechnology and nanotechnology may facilitate the development of pesticides with nominal adverse effects. Community development and extension

programs that could train and motivate the farmers to take up the contemporary integrated pest management (IPM) strategies may reduce the negative impact of pesticides to our environment which may lead to discontinuing our anthropogenic legacy of silent spring.

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Compliance with Ethical Standard

Conflict of Interest: There is no conflict of interest between any authors to publish this review article.

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Chapter 4
Genetically Modified Microbial Biosensor for Detection of Pollutants in Water Samples

Sunantha Ganesan and Namasivayam Vasudevan

Contents

Abstract Persistent organic pollutants including perfluorinated compounds, polyaromatic hydrocarbon, polychlorinated biphenyls, pesticides, and heavy metals are released into the environment due to rapid industrialization and anthropogenic activities. As a result, groundwater and surface water contamination has become a major threat to human and aquatic organisms. Analytical techniques such as high performance liquid chromatography, liquid chromatography–mass spectrometry, gas chromatography, gas chromatography–mass spectrometry, atomic absorption spectroscopy, and inductively coupled plasma optical emission spectrometry are used for monitoring persistent organic pollutants and heavy metals, and these techniques are

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expensive, sensitive, and labour intensive, and it requires sample purification step before chromatographic analysis. Microbial biosensors are the promising approaches for the detection of hazardous compounds. In this chapter, bacterial biosensors for identification of persistent organic pollutants and heavy metals in water samples are discussed in detail.

Keywords Biosensor · Chromatography · Pollutants · Heavy metals · Persistent organic pollutant · Reporter protein · Genetically modified biosensor · Gas chromatography · High performance liquid chromatography

4.1 Introduction

The rapid urbanization and industrialization increases pollutants in the environment. They form diverse collections of chemical substances, such as pharmaceuticals (human, veterinary, and illicit), personal care products (diethyltoluamide, parabens, and triclosan), flame-retardants (triazole, alkyl phosphates, and polybrominated diphenyl ethers), pesticides (parathion and chlorpyrifos), hormones (estradiol and cholesterol), surfactants (Alkyl ethoxylate), and industrial additive agents as well as their transformation products. These substances have been detected in surface water, drinking water, groundwater, sludge, soil, sediment, aquatic environment, atmosphere, fish, and human blood can be contaminated with nanogram to milligram/litre. Figure 4.1 illustrates the sources and exposure of organic pollutants.

4.1.1 Pollutants: Introduction and Threats

There are thousands of persistent organic pollutants (POPs), which include aldrin, chlordane, heptachlor, hexachlorobenzene, dioxins/furans, phthalates and dichlorodiphenyltrichloroethane, and endosulfan (alpha and beta), often coming from certain series of chemicals. In 2009, perfluorinated (particularly, perfluorooctanoic acid (PFOA) and perfluorooctane sulphonate (PFOS)) compound has been classified under persistent organic pollutants, and these compounds differ from each other by the level of the fluorine atom and its position (Post et al. 2012). The main sources of these pollutants in the environment are due to disposal of agrochemicals and industrial chemicals and improper management of solid waste in developed and developing countries. Persistent organic pollutants remain in the environment and accumulate in human tissues. Therefore, they may create impact on human health and cause adverse and toxic effects that inhibit human intercellular communication and deoxyribonucleic acid (DNA) damage (Wilson et al. 2016). The World Health Organization (WHO) guidelines recommended for the human exposure on permissible limits and toxicity of persistent organic pollutants are given in Table 4.1. The WHO and United

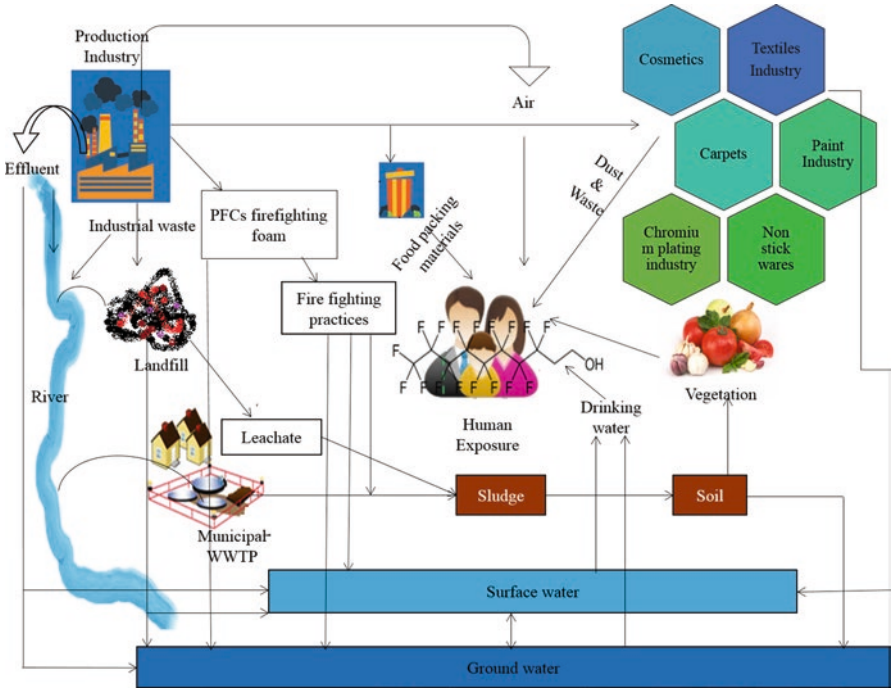

Fig. 4.1 Sources of major organic pollutants in the environment

State Environmental Protection Agency (US EPA) estimate that most of the diseases occur due to prolonged exposure to environmental pollution.

4.2 Methods for Quantification/Identification of Pollutants in Environment

High performance liquid chromatograph (HPLC) connected with ultraviolet/fluorescence detector, gas chromatography coupled with mass spectrometer (GC-MS), electron capture detector, and flame ionization detector with various means of detection and different types of spectroscopy (atomic absorption and atomic emission spectroscopy) are commonly used analytical techniques for detection of environmental pollutants (APHA 2005). The chromatographic techniques required tedious purification to reach detection limits of the analytes present in the environment.

Various research articles have highlighted the importance of sample preparation and detection limit of pollutant (Demeestere et al. 2007; Bellan et al. 2011; Petrovica et al. 2010; Martin et al. 2004; Poothong et al. 2013). Most of these analytical techniques are quite complex with extraction steps either through solid or liquid phase

Table 4.1 Permissible limits of consumption and effects of the persistent organic pollutants to human health

Pollutant	Permissible limit	Detection limits for analytical techniques	Target effects	References
Polychlorinated biphenyls hotspots				
Seveso disaster was an industrial chemical plant explosion that occurred in Lombardy region of Italy (1976). It resulted in the known exposure to 2, 3, 7, 8-tetrachlorodibenzo dioxin in residential area.				
Times beach (United States) is completely evacuated in 1983 due to dioxin contamination. It was the largest civilian exposure to dioxin in the world.				
Polychlorinated biphenyls	1 ng/L	<1 μg/L (GC)	Thymus and nervous problem, risk of cancer, and immune deficiency	WHO (2003a, b)
Dichloro diphenyl trichloroethane	40 μg/L (Food) <46 ng/L (Water) <60 μg/L (Air)	60 ng/L (*P*,*P*-DDT), 10 ng/L (*P*,*P*-Dichloro diphenyl dichloro ethylene), 2.5 ng/L *P*,*P*-Dichloro diphenyl dichloro ethylene (GC)	Cancer risk, affected psychomotor or mental behavioral patterns	WHO (2004a, b, c)
Pesticide Hotspots				
An explosion at a Union Carbide pesticide plant in Bhopal, during 1984, led to the worst industrial accident in history. At least 2000 people died, and another 200,000 were injured when methyl isocyanate and other toxic gas enveloped in the city.				
The Sandoz spill (1986 at Switzerland) was major disaster, which released toxic agrochemicals into the environment.				
Endosulfan (α and β, endosulfan sulphate)	–	0.015 μg/L and 0.024 μg/L for α and β endosulfan, 0.015 μg/L for endosulfan sulphate	Convulsions, psychiatric disturbances, paralysis, and endocrine disruption	WHO (2004a, b, c)
Atrazine	–	0.1 μg/L (GC)	Cardiovascular and reproductive problems	WHO (2010)
Chlorpyrifos	–	0.1 μg/L (GC- flame thermionic or photometric)	Mammalian cell and neural disorders	WHO (2004a, b, c)
Polyaromatic hydrocarbons hotspot				
Jilin chemical plant explosions (2005) in China are due to petrochemical plant explosion, benzene, and nitro benzene passed into Amur River.				
Polycyclic aromatic hydrocarbons	1 ng/L	0.01–200 ng/L (GC)	Developmental and reproductive effects	WHO (2003a, b)

(continued)

Table 4.1 (continued)

Pollutant	Permissible limit	Detection limits for analytical techniques	Target effects	References
Perfluorinated compounds				
Perfluorooctanoic acid	70 ppt	1 ng/L (Liquid chromatograph- mass spectrometer)	Reproductive, developmental toxicities and etc.	US EPA (2014, 2016)
Perfluorooctane sulphonate				

methods. These methods are time consuming and laborious (Rodriguez-Mozaz et al. 2007). Additionally, these instruments need column cleanup processes. Based on this, biosensor systems have shown tremendous promise to overcome these limitations (Rodriguez-Mozaz et al. 2005). This biological technique includes immuno- and bioassay method. Microbial biosensors have become one of the popular molecular tools for monitoring of persistent organic pollutants.

4.3 Biosensor

4.3.1 Definition of Biosensor

A sensor has transducers to sense pollutants in the environment. It detects and provides a corresponding output, generally as an electrical or optical signal. It has an ideal alternative method for pollutant quantification, as they are the most effective technique (Turner 2007). The term "biosensor" is in short for "biological sensor". A "biosensor" is a self-contained integrated device, consisting of a biological recognition element in direct contact with a transduction element, which converts into a measurable output signal resulting in qualitative or quantitative identification. The transducer converts this biological signal into a quantifiable signal (potential, optical, or electronic signal) which can be further improved, processed, and stored for later analysis (Kaur et al. 2015; Teo and Wong 2014).

4.3.2 Types of Biosensor

Biosensors have been classified based on transduction and biological recognition elements (Fig. 4.2). Biosensors have been constructed based on the interactions between antibodies and antigen, enzymes and their substrates, or the affinity of nucleic acid strand to their complementary sequences. Biosensor selectivity and specificity are highly depended on biological recognition systems connected to a suitable transducer. Recently, biosensor researchers are highly attracted by recognition molecules such as phages, molecular imprinted polymers, and affibody that are

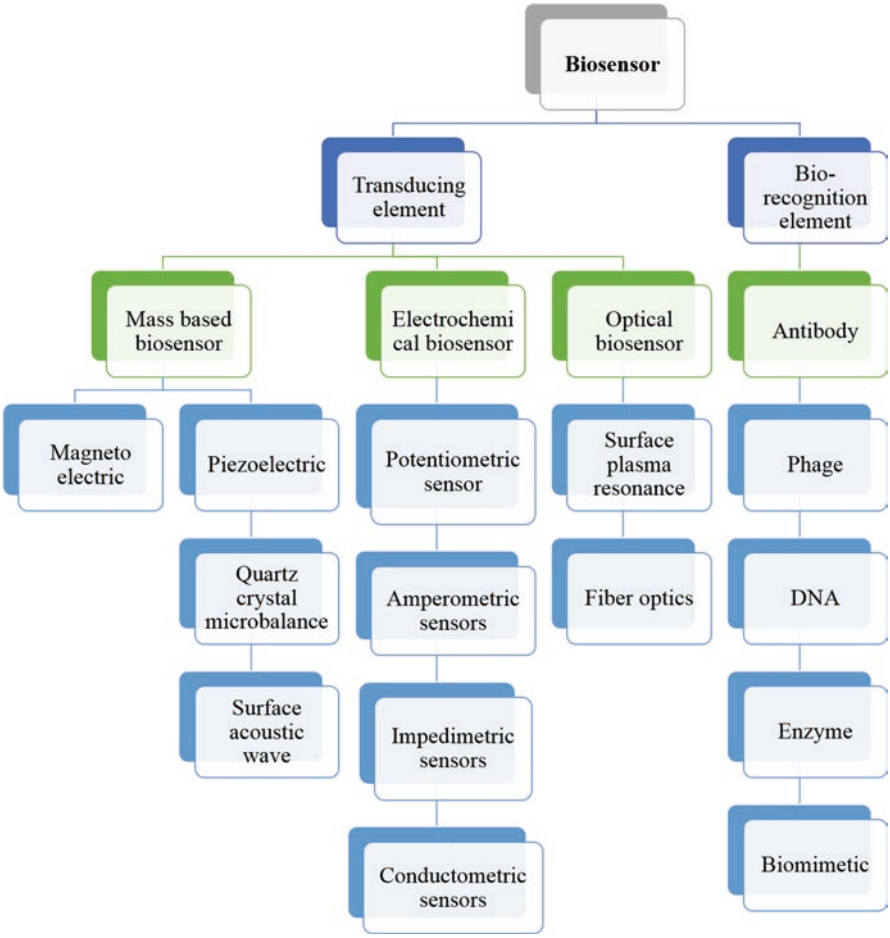

Fig. 4.2 Classification of biosensor based on the transducer and biorecognition element

used for the development of biosensors with improved performance (Dorst et al. 2010; Perumal and Hashim 2014; Justino et al. 2015).

4.4 Bacterial Biosensor

Microorganism, specifically bacteria, shows potential strategy for developing bacterial biosensor that has various advantages. Since bacteria can be cultured in liquid media and have faster growh rate, *E. coli*, *Bacillus and Pseudomonas* are used for the construction of bacterial biosensors. Divies developed the first microbial (*Acetobacter xylinum*) biosensor in 1975 for detection of ethanol (Divies 1975).

This work became a base for developing microbial biosensors for pollutant monitoring purposes. Most of the sensors developed till date fall under the taxonomic group of microbes. This is due to rich analytical prospects of the microbes and their reliability.

The development of sensor requires the immobilization of biorecognition element; it secures both the stabilization and proximity of the recognition element for reuse. The immobilization plays a key role in developing stable biocomponent for integration with transducers (Rodriguez-Mozaz et al. 2005). The immobilization techniques are physical adsorption, cross-linkage, covalent binding, and entrapment method. Selection of immobilization methods would depend on the nature of the biomaterial and configuration of the transducer used. Cross-linkage and covalent binding techniques are not suitable for microbial cells; it affects the viability and may loss structural integrity, but applied for immobilization of antibodies/antigens and the enzymes.

Natural polymers used for the entrapment of the cells include carrageenan, alginate, chitosan, and low-melting agarose. These polymers are known to be very useful in obtaining viable cell-immobilized systems. Among these, entrapment in alginate by ionotropic gelation using a variety of divalent and trivalent cations has found extensive use in immobilization (Gupte and D'Souza 1999). However, alginate and carrageenan have strong metal absorption capacities. There are many different types of microbial biocomponent (from disposable to reusable) by immobilizing microbial cells on different supports and connected to transducers (Kumar and D'Souza 2010, 2011; Jouanneau et al. 2012; Roda et al. 2011; Charrier et al. 2010). Kumar and D'Souza (2010) reported an optical biosensor with immobilized bacteria *Sphingomonas* JK1 on the bottom side of the microplate and linked with an optical plate. This system enables multiple sample detection on one plate. Biosensor can detect 4–80 μM of methyl parathion and can be reused up to 75 times.

Jouanneau et al. (2012) reported an online biosensor analysis system for continuous monitoring of arsenic (As), cadmium (Cd), mercury (Hg), and copper (Cu) in water by developing two bacterial biosensors, namely, Lumisens III and Lumisens IV. In the first Lumisens, bioreporters were immobilized in agarose hydrogel in a multiwall card, and in the second one, freeze-dried bacteria were utilized. The Lumisens IV system was more successful in terms of stability, sensitivity, time consumption, and reproducibility (40%). Elad et al. (2011) reported a chip-based analysis system for continuous monitoring of heavy metals including arsenic and antimony in water samples in laboratory conditions with a reproducibility of up to 20%.

4.5 Genetically Modified Biosensor

The genetically modified bacterial biosensor for specific pollutant appears to be easily achieved by various molecular techniques. Metabolic and protein engineering has enhanced the production of biochemical signals. The genetically modified biosensor contains two genetic elements, namely, regulatory and reporter gene.

Regulatory gene usually emerges from microorganisms that are resistant to a specific pollutant. Using genetic engineering, a promoter sequence from one microbial species can be fused to a reporter gene from other microbes. The fused protein can be introduced into bacterial chromosome, and it allows better stability of the system. The regulatory gene is activated by specific environmental condition; the activation would lead to synthesis of proteins, the activity which helps the cell combat the hazardous material or adapt it. It is important to note that the detection of environmental molecules using microbial biosensor is generally based on assays where the expression of the reporter gene is either inducible or constitute.

Green fluorescence protein, luciferase, and β-galactosidase are used as a reporter protein in genetically modified biosensor (Table 4.2). One of the most popularly and commonly used reporter genes is known as green fluorescence protein. This is due to easy availability, the lack of requirements for a substrate or the co-factor, and the ability to use single-cell detection. Figure 4.3 represents the bacterial biosensor for detection of specific pollutants; specific pollutant is identified by regulatory gene, which then activates the promoter attached to the reporter gene. The transcription is initiated in the synthesis of a regulatory protein and produces the measurable signal.

4.5.1 Genetically Modified Bacterial Biosensor for Persistent Organic Pollutants

As discussed earlier, pollutants are a serious problem in both developed and developing countries. It is necessary to monitor pollutants in the environment. To detect persistent organic pollutants, genetically engineered microbial biosensors have been developed by various researchers. Table 4.3 summarizes the different types of bacterial biosensors for the detection of persistent organic pollutants and heavy metals.

4.5.2 Genetically Modified Bacterial Biosensor for Polychlorinated Biphenyls

Polychlorinated biphenyls are synthetic organochlorine compounds, which contain multiple no. of chlorine atoms with a wide range of industrial applications due to its chemical and thermal stability. Polychlorinated biphenyls are most widely identified xenobiotic compounds in the environment. Leedjarv et al. (2006) constructed a biosensor to determine the phenols in leachate site and groundwater. In this study, phenol's (methylphenols, dimethylphenol, and resorcinol) availability was assessed by transcriptional fusion (*DmpR-Po-lux* CDABE) between regulatory (*DmpR*) and reporter gene (*lux*). The sensor was able to detect phenols with 4 h incubation and detectable limit reached up to 0.08 mg/L for phenol and 0.03 mg/L for methyl

Table 4.2 List of marker genes used for biosensor construction

Gene	Origin	Potential substrate	Assay	Advantages	Limitation	References
Green fluorescence protein *(gfp)*	*Aequorea victoria*	No need substrate	Fluorescence	Autofluorescence	Low sensitivity	Lewis et al. (2000)
β-galactosidase (*lacZ*)	*E. coli*	β- Galactosidase	Colorimetric	Detection by naked eye	Substrate required	Close et al. (2010)
Luciferin (*Lux*)	*Vibrio fischeri*	Luciferin, adenosine triphosphate (ATP)	Luminescence	Easy of measurement and rapid response	Heat labiality and oxygen required	Myronovskyi et al. (2011)
Luciferin (*Luc*)	*Photinuspyralis*	Luciferin, ATP	Luminescence	High sensitivity	Substrate required	Allard and Kopish (2008)
Chloramphenicol Acetyl CoA (*Cat*)	*E. coli*	Chloramphenicol Acetyl CoA	Thin layer chromatography	No endogenous activity	This assay relies on the radioisotopes	Wilson and Hastings (1998)
Aequorin	*Aequorea victoria*	Calcium binding protein	Luminescence	High sensitivity and no endogenous activity	Requires substrate	Yagi (2006)
Uroporphyrinogen III methyl transferase	*Bacillus megaterium* and *P. denitrificans*	No substrate requirement	Fluorescence	Auto fluorescent	Endogenous activity	Sattler et al. (1995)

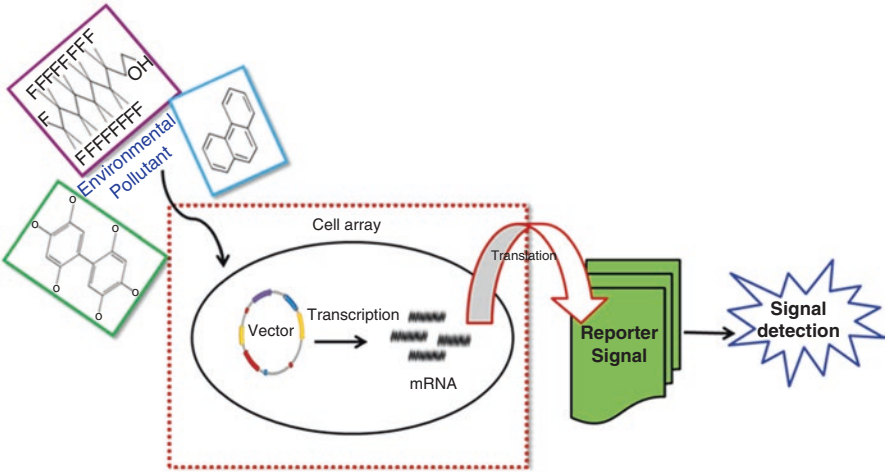

Fig. 4.3 Schematic representation of bioregulator bacterium for specific pollutant

phenol. In a study, Turner (2007) developed a whole-cell biosensing system for the detection of polychlorinated biphenyls by employing the strain *Pseudomonas azelaica* HBP1. This bacterium contains the *hbpCAD* genes that are responsible for the degradation of hydroxylated biphenyls. A regulatory protein encoded by the gene hbpR located upstream from the hbpCAD genes regulates the expression of these genes. A strain of *E. coli* carries a recombinant plasmid consisting of *luxAB* reporter gene under the control of HbpR regulatory protein. The detection limits ranging from 1×10^{-5} M to 1×10^{-9} M depend on which *para*-substituted polychlorinated biphenyls. Similarly, soil bacterium *Ralstonia eutropha* ENV307 (pUTK60) is used for monitoring the polychlorinated biphenyls by inserting the biphenyl promoter (*bphA1/BphS*) upstream of the bioluminescence gene (*lux* CDABE). The minimum detectable limits for these compounds ranged from 0.15 mg/L for 4-chlorobiphenyl to 1.5 mg/L for Aroclor.

Bhattacharyya et al. (2005) developed a different set of constitutive and inducible bioreporters to detect chlorinated aliphatic hydrocarbons in groundwater. The modified *Pseudomonas putida* TVA8 linearly responds to trichloroethylene. A detection limit of sensor system was achieved up to 2000 µmol/L. Later, Tecon and Van der Meer (2008) constructed a biosensor for detecting aromatic hydrocarbon. The *Burkholderia sartisoli* RP007 (pPROBE-*phn-luxAB*) strain detects naphthalene and phenanthrene in seawater. The modified strain was able to detect hydrocarbon in seawater with 3 h incubation and detection limit reaching up to 0.17 µM. From this study, the biosensor could be used as a simple and rapid tool for identifying hydrocarbons in oil-contaminated sites. Ivask et al. (2001) developed a bacterial sensor system for detection of methyl mercury compounds. The sensor carried *lux* gene as a reporter gene under the control of mercury-inducible regulatory protein (*mer* from *Serratia marcescens*). The sensitivity of the sensor was tested on some important organomercurial compounds. The lowest detectable concentration was

Table 4.3 List of selected examples of genetically modified microbial biosensors for detection of environmental pollutants

Pollutants	Gene cassette	Detectable output	Detection range	Frames	References
Phenol	*f*abA and *f* abR	β- galactosidase	1.6–16 ppm	*E. coli*	Hillson et al. (2007)
2,4-Dichlorophenoxyacetic acid	*EfdRP*$_{DII}$*lux CDABE*	Bioluminescence	0.25 mg/L	*Rastonia eutropha*	Neufeld et al. (2006)
Alkyl sulphonates	*Hao-luxAB*	Bioluminescence	–	*Nitrosomonas europaea*	Toba and Hay (2005)
Toluene analogues	*P*$_m$*-lux*	Bioluminescence	0–5 mM	*P. putida* mt-2 KG1206	Brandt et al. (2002)
Atrazine	*tac-luc-luxAB-aphII*	Bioluminescence	7.39 mg/L	*Synechocystis* sp. PCC6803	Kong et al. (2007)
Propazine			3.51 mg/L		
Simazine			1.06 mg/L		
Methyl mercury	*E. coli* with *luc*	Bioluminescence	50 ng/L	*E. coli*	Shao et al. (2002)
Dimethyl mercury			2.34 mg/L		
Phenolic compounds	*capR- lux CDABE*	Bioluminescence	0.1–10 mM	*E. coli*	Ivask et al. (2001)
Naphthalene	*nahG- lux CDABE*	Bioluminescence	0.5 mg/L	*P. fluorescence*	Shin et al. (2005)
Pesticides	*E. coli* linA2 and LinA2	Pulsed Amperometric	2–45 ppt	*E. coli*	Heitzer et al. (1994)
As	*arsR/luxAB*	Bioluminescence	4–7 ng/L	*E. coli* DH5	Trang et al. (2005)
Hg	*merRT-luxCDABE*	Luminescence	0.5 ng/L	*E. coli* JM109	Larose et al. (2011)
Cu	*luxAB*	Bioluminescence	300 µg/L	*P. fluorescens* DF57-Cu15	Tom-Petersen et al. (2001)
Cd	*cadR-crtI/cadR-lacZ*	Red pigment	1–10 mM	*Deinococcus radiodurans*	Joe et al. (2012)
Cu and Zn	*cusC-gfp/rfp; zraP-gfp/rfp*	Fluorescence	26 µM (Cu);16 µM (Zn)	*E. coli* XL1-Blue	Ravikumar et al. (2012)
Ni^{2+} and Co^{2+}	*cnrYXH-luxCDABE*	Bioluminescence	9 µM (Ni^{2+}); 0.1 µM (Co^{2+})	*Ralstoniaeutropha*AE2515	Tibazarwa et al. (2001)
As	*arslux CDABE*	Luminescence	0.74–60.00 µg/L	*E. coli*	Sharma et al. (2013)

observed at 50 ng/L for methyl mercury chloride, 0.34 μg/L for phenyl mercury acetate, and 2.3 mg/L for dimethyl mercury. Willardson et al. (1998) developed whole-cell biosensor system to sense toluene, and the results were cross-checked with GC-MS.

4.5.3 Genetically Modified Bacterial Biosensor in Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons are found ubiquitous in the environment due to natural and anthropogenic activities. The anthropogenic activities include petrogenic and pyrogenic activities. It occurs in the environment due to incomplete combustion of fossil fuels, burning of wood and coal, and the natural activities including volcanic eruption, forest fires, etc. Polycyclic aromatic hydrocarbons are mutagenic and carcinogenic. Soil can be contaminated with 1 μg/kg to 300 g/kg of polycyclic aromatic hydrocarbons. There are various methods used for the detection of polycyclic aromatic hydrocarbon, but still they have some limitations. Kuncova et al. (2011) reported *P. putida* TVAS with a *tod-luxCDABE* is sensitive for the bioavailability of organic pollutants. The bioreporter was responsive to 23 organic pollutants, whereas *tod* reporter mostly identified benzene, ethylbenzene, xylene, and toluene from wastewater and groundwater samples with 0.5–120 mg/L detection limit. Using similar strategies, Kohlmeier et al. (2008) reported a highly sensitive naphthalene bioavailability sensor based on a *nahR-gfp* fusion in *P. putida*. The strain was applied for the detection of naphthalene in water and gas phase. It turned out that the detection limit of the compound is 50 nM in gas medium and 0.5 μM in water. Shin (2010) reported that the mutagenesis of the effector binding sites of regulators involved in the degradation of naphthalene, and salicylate increases the sensitivity and specificity to polycyclic aromatic hydrocarbons. To increase the sensitivity of the biosensor, eight single mutants (N169A, N169C, N169K, N169S, R248H, R248M, R248Q, and R248Y) were made at residues 169 central inducer recognition domain and 248 C-terminal multimerization domains in *nahR* regulatory gene. The substitution of the amino acids leads into drastic changes in the microbial response to salicylate, including the increase sensitivity of biosensor. The effects of these mutations were examined by monitoring expression of a *luc* reporter gene under the control of *nahR*. The biosensors showed response to toxic concentration up to 5 mM. Thus, the mutagenesis method could be overcoming the draw of low sensitivity in developing microbial biosensor.

4.5.4 Genetically Modified Bacterial Biosensor in Pesticides

Pesticides are synthetic organic compounds, and EPA restricts application of many pesticides (Endosulfan) due to environmental concern and human health. The recalcitrance of pesticides causes serious problems. It leads to immunotoxicity, carcinogenicity, and damage to hormonal imbalance. Pesticides have been detected in soil, water, and surface sediments. Serdar et al. (1989) detected organophosphorus pesticides using genetically engineered *E. coli* harbouring the organophosphorus degradation gene of *Pseudomonas putida*. A whole-cell electrochemical biosensor was developed for detection of organochlorine pesticide using pulsed amperometry with a detection limit of 2 ppt (Prathap et al. 2012). By the expression of *linA2* gene encoding the γ-hexachloro cyclohexane dehydrochlorinase from *Sphingomonas paucimobilis, lindane was* degraded with the associated generation of hydrochloric acid in *E. coli* immobilized on a polyaniline film. This electrochemical biosensor provides a more suitable platform for on-site monitoring of environmental samples as purified γ-hexachlorocyclohexane dehydrochlorinase that is likely to be inhibited by several physicochemical factors such as organophosphate pesticides. In another study, Kim et al. (2013) developed a biosensor for monitoring paraoxon. This sensor system showed long-term stability (28 days) with 80% retained activity and reusability for up to 20 times. Several evidences confirmed the sensitivity of genetically modified microbial biosensors toward environmentally toxic compounds.

4.6 Genetically Engineered Bacterial Biosensor for Heavy Metal Detection

Industrial and human activities are increasing the heavy metals (atomic weight between 63.5 and 200.6 g mol^{-1} and a specific gravity greater than 5 g cm^{-3} are called as heavy metals) in the environment; Cd, Hg, and lead (Pb) are of considerable interest due to their toxicity and pollution issue.

A sensor plasmid was constructed by inserting the regulation unit to control the expression of reporter gene. Modified *Staphylococcus aureus* strain RN4220 (pTOO24) mainly responded to cadmium, lead, and antimony. The detection limit reached up to 10 nM, 33 nM and 1 nM, respectively. Modified *Bacillus subtilis* strain BR151 (pTOO24) responds to Cd, antimony, zinc, and tin. The response was achieved with 2–3 h incubation (Tauriainen et al. 1998). Further, Ivask et al. (2004) constructed a biosensor to examine the bioavailability of Cd and Pb in soil. In this work, they have constructed two recombinant bacterial biosensors (*lux* used as reporter gene) for identifying Cd and Pb in agricultural soils nearby metal smelter

site. Biran et al. (2000) developed sensor system for monitoring the Cd in seawater and soil samples. The Cd-stress responsive gene is fused to a *lacZ* gene and observed the Cd concentrations in soil samples. Ivask et al. (2009) developed multiple number of recombinant bacterial biosensor for testing heavy metals and general toxicity. In this study, they have used 19 recombinant bacterial strains representing various group of gram-positive and gram-negative bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *E. coli*, *P. fluorescens*, respectively) and were constructed to express the luminescence gene (*lux* CDABE) as a response to heavy metals in water.

Tibazarwa et al. (2001) employed the luminescent bacterium *Ralstonia eutropha* (AE2515) harbouring the *lux* gene to study bioavailable concentrations of nickel (Ni) and cobalt (Co) in soil. This plasmid (pMOL1550) carried *cnrYXH* regulatory genes that are transcriptionally fused to *luxCDABE* reporter system. Strain AE2515 was standardized for its specific responses to Co and Ni. The detection limits of the biosensor were 0.1 μM for Ni and 9 μM for Co, respectively. Another study reported the detection of As in water with 0.5–2.5 h incubation and detection limit recorder as 7 mg/L (Trang et al. 2005). A recombinant *Staphylococcus aureus* strain was developed for As analysis. The strain was found to be more stable (6 months), simple, and sensitive, and the detection limit reached up to7.5 mg/L (Tauriainen et al. 1999). Ravikumar et al. (2012) designed and applied biosensor for zinc (Zn) and copper (Cu) in water samples. The P and *cus*C promoters were fused to a dual-labelling reporter protein as an interactive biocomponent for zinc (Zn) and copper (Cu) with the detectable limit of 16 μM and 26 μM for Zn and Cu, respectively, and the sensor system proved sensitive and an effective technique for detection of Zn^{2+} and Cu^{2+} in field water samples.

Aleksic et al. (2007) reported biobrick sensor system (*E. coli* JM109/pSB1A2-BBa-J33203) for As identification. The system was found to be a clear response to arsenate concentrations as low as 5 ppb in environmental samples. Roointan et al. (2015) developed a biosensor for the detection of Hg in liquid solutions. The sensor carries *gfp* as a reporter gene under the control of Hg-inducible regulatory gene from *Pseudomonas* pBS228 which was cloned into pUC19 vector and transferred into Escherichia coli (*E. coli*) BL21 strain. The sensitivity of the sensor was evaluated with Hg metal. Modified biosensors respond to Hg (II), with detectable limit reaching up to 10^{-8} M for 3 h incubation. The *E. coli* DH5α strain harbouring the *lux*AB reporter gene from *Vibrio harveyi* transcriptionally fused with *ars*R reported gene (*E. coli* DH5 α (pJAMA-*ars*R)) for monitoring As in groundwater. The developed biosensor was also reported to have the presence of phosphate, silicate, and iron and produces luminescence (Harms et al. 2005). To overcome this issue, recombinant DNA can help to enhance the specificity of microbial sensors by activating specific pathways of metabolism and cellular uptake while switching off the undesirable's gene action.

Wu et al. (2009) have improved the sensitivity of microbial biosensors via inserting additional repressor gene to reduce the background fluorescence by nonspecific inducers such as isopropyl β-d-1-thiogalactopyranoside and increase the sensitivity of the cells toward Cd. Another limitation which includes widespread use of microbial biosensors is the inherent difficulty of maintaining cell viability and activity in

complex environments that may lack nutrients and may contain inhibitory compounds. In this regard, Close et al. (2010) highlighted that the challenges in the development of microbial biosensors are to keep the whole cells viable through a long storage and to immobilize the cells tight and close to the transducers.

4.7 Risk and Regulations of Genetically Modified Organisms

Advanced biotechnological techniques have enabled the construction of microbes through genetic modification with improved novel functions. These genetically modified organisms have the potential role in various fields including agriculture, bioremediation, environmental management, aquaculture, and forestry. Genetic modification has the potential to confer improved tolerance or resistance to various stresses and consequently improve the performance of the degradation of persistent organic pollutants. Currently, biosensors are being used to monitor and degrade various toxins in the environment. Though the benefits of genetically modified organisms have many positive outcomes, accidental or deliberate release of genetically modified organisms into the environment could have negative impacts on the environment.

4.8 Limitation of Genetically Modified Bacterial Biosensor

Genetically modified microbial biosensors are most powerful and versatile tool than other type of biosensors. However, there are several limitations for constructing genetically modified biosensors such as interference by high background environmental pollutants; this leads to locking of selectivity.

The risks related to release of genetically modified organisms in the open environment are as follows:

- There is little prior experience about the combination of host and modified organisms.
- The genetically engineered microorganism may persist and proliferate the host without human intervention.
- Genetic exchange is possible between the wild-type and transformed organism.
- The improved traits of transformed organism confer an advantage over the native species.

In order to reduce potential risk of genetically modified organisms in the environment, some genetic barriers are created. The spread of genes from genetically modified organisms to other microorganisms may be limited by using transposons without transposon gene or by removing conjugation genes from the recombinant plasmid. Random horizontal gene transfer can be also diminished by inserting into vector colE3 gene encoding colicin that cuts all prokaryotic 16S rRNA (ribosomal

ribonucleic acid) and by controlling immE3 gene encoding repressor of colicin synthesis (Davison 2005).

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Chapter 5
Integration of Polyhydroxyalkanoates Production with Industrial Wastewater Treatment

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Contents

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Abstract Plastics have been a revolutionary material in every aspect of human life since their development, but have also led to one of the leading environmental issues with regard to their disposal. These petrochemical-derived plastics take up to 2000 years to break down in the environment and accumulate within living organisms. A potential alternative is to replace petroleum-based plastics by biodegradable ones. Polyhydroxyalkanoates are one of the most promising types of biodegradable polymers with properties similar to polypropylene and are readily produced by more than 90 known species of bacteria as intracellular polyester stores. The most widely employed production technique for polyhdroxyalkanoates is by single-organism fermentation processes under a feast-famine regime. However, the high cost of sterilization and substrates typically employed for industrial production means commercial production is limited for niche polymer applications. Industrial wastewater provides an alternative low-cost carbon feedstock that can provide benefits of combined organic treatment and polyhydroxyalkanoates production. Such combination precludes single-organism systems, meaning microbial mixed cultures must be used.

This chapter explores the limitations and factors essential for successful microbial mixed culture cultivation and polyhydroxyalkanoates production coupled with industrial wastewater treatment. The specific target operating conditions will differ depending on the specific wastewater substrate and target microorganisms for enrichment. A high degree of cellular storage is important for high productivity and economic processing and can be induced through various redox conditions, specific feeding regimes, use of nutrient limitation, specific pH control, and, in the case of photosynthetic cultures, light intensity, all of which influence the specific microorganisms enriched. The specific enriched microorganisms in conjunction with the substrate are the primary factors defining the monomer compositions of the polyhydroxyalkanoates, which infers its mechanical properties and potential application. Use of microbial mixed cultures compared with single-organism cultures makes control of polyhydroxyalkanoates composition more challenging, but provides greater resilience to wastewater composition and flow fluctuations due to community redundancy and microbial succession. The most significant drawback, however, of microbial mixed culture use with industrial wastewater treatment is the downstream processing of more dilute cell concentrations than single-organism processes. Further research into both cell concentrating and polyhydroxyalkanoates extraction techniques will help improve economics and sustainability of the process. Nevertheless, initial theoretical assessments show that development of commercial polyhydroxyalkanoates production from industrial wastewater using microbial mixed cultures should be cost competitive and more sustainable than either petrochemical plastics or polyhydroxyalkanoates produced by single-organism fermentation.

Keywords Biopolymers · Intracellular storage · Circular economy · Resource recovery · Mixed microbial culture · Bioplastic · Feast-famine · Polyhydroxybutyrate (PHB) · Substrate · Redox

5.1 Background and Introduction

5.1.1 Plastic Production, Disposal, and Sustainability

In our daily life, we are surrounded by plastic materials. Whether it is plastic bags for food packaging, furniture and appliances, or disposable containers, they have become an indispensable material in our day-to-day activities due to their light weight and durability. While plastics have dramatically improved and simplified our lives, the use of plastics for packaging and other purposes has increased the issue of solid waste disposal (Salehizadeh and van Loosdrecht 2004). Plastic use and polymer production have grown steadily for the past 50 years increasing from 15 million tons in 1964 to 311 million tons in 2014 (World Economic Forum 2016).

These vast quantities of polymer products require disposal or recycling at the end of their life cycle. It was estimated in the 1990s that plastics were accumulating at a rate of 25 million tons/year (Salehizadeh and van Loosdrecht 2004). This rate is expecting to increase until 2020 (Castilho et al. 2009). While the majority of disposed plastic products are recyclable, around 22–43% of polymers disposed annually end up in landfills, which have low degradation rates and can potentially lead to groundwater pollution by leaching out toxic additives. Incineration is also used; however, it is an expensive option associated with harmful air emissions (Castilho et al. 2009). Only 9% of plastics are recycled properly, while the rest are not recovered. Moreover, when these polymer wastes enter the environment, they may take up to 2000 years to break down (Dietrich et al. 2017). This results in an estimated eight million metric tons/year of accumulation (Jambeck et al. 2015), with an estimate of 100 million tons in the Pacific Ocean alone (Marks and Howden 2008). As a consequence, it is estimated that more than a million aquatic animals are killed yearly by choking on or ingesting plastic debris (Rodriguez-Valera 1991). In addition to disposal issues, the vast majority of polymers are currently produced from petroleum feedstocks, consuming currently around 6% of total petroleum use and estimated to rise 20% by 2050 (World Economic Forum 2016). The conversion processes typically involve high heat and pressure resulting in significant energy consumption and carbon dioxide emissions (National Research Council 1994). In light of growing concern over global warming, international agreements to mitigate greenhouse gas emissions and efforts to slow fossil reserve depletion, there is a developing interest from industry and consumers toward alternatives.

Bioplastics have been suggested as an environmentally friendly alternative to petrochemical plastics. Bioplastics are polymers that are either (1) biobased, meaning derived from renewable biomass resources such as agricultural feedstocks, (2) biodegradable, meaning bioplastics that degrade naturally in the environment, or (3) biobased-biodegradable, which meet both criteria and are the most desirable (Fig. 5.1). However, with respect to agricultural feedstocks, concerns still exist, similar to biofuel crop production, over potential competition with food crops for land and water resources (Detzel et al. 2013). Therefore, use of waste residues as feedstock, where compatible with the bioplastic production process, is a more

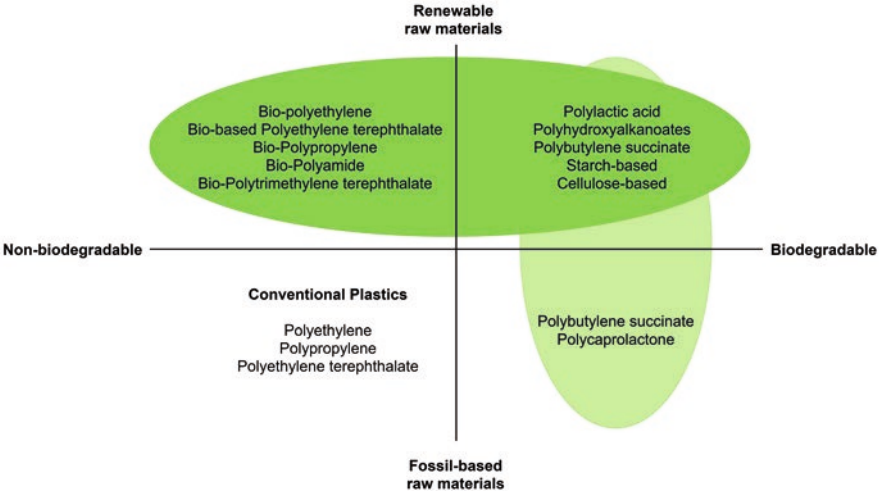

Fig. 5.1 Types of bioplastics and classification based on their material source and biodegradability

sustainable route. The biodegradable bioplastics decompose into carbon dioxide and water in the environment after their disposal. While the biobased bioplastics are composed of renewable raw material, they do not necessarily decompose after their disposal. The biodegradable biobased bioplastics can be derived from starch-based, cellulose-based, or polyester sources. Polylactic acid is one of the most widely used types of biodegradable biobased bioplastics. Bio-polyethylene, bio-propylene, and polybutylene succinate are all potential replacements for petroleum-based plastics (Chee et al. 2010). The biodegradable petroleum-based plastics include polycaprolactone and poly(butylene adipate-co-terephthalate). However, they are not common (Bugnicourt et al. 2014; Kourmentza et al. 2017).

Currently, only 1% of all polymers in use are biopolymers, although the industry is slowly but continuously developing (Dietrich et al. 2017). About 2.05 million tons of bioplastics were produced in 2017. This number is expected to increase to 2.44 million tons by 2022 (European Bioplastics 2017). Additionally, the increasing negative impact on human health from petrochemical plastics is leading more people and companies to become more environmentally aware (Rodriguez-Perez et al. 2018). A number of governments are beginning to ban single-time-use plastics and encouraging circular economies instead. For instance, Italy banned petroleum-based plastic bags back in 2011 and is now ranked as the largest global consumer of bioplastics. France has recently banned plastic cutlery and cups, which should be in full effect by 2020 (Vandi et al. 2018). Many multinational companies are following the recycling and bioplastic trends. Examples include Starbucks, who have recently banned straws from their US stores (Starbucks Coffee Company 2018), LEGO which has invested millions of dollars into research to produce LEGO bricks from bioplastics (LEGO 2018), and IKEA who expects to be using recycled plastics and bioplastics only by 2020 (Neste Corporation 2018). Others include The Body Shop

who have now made all cosmetic packaging from polyhydroxyalkanoates, a bioplastic derived from fermentation or plants (Vandi et al. 2018), and Coca-Cola, who is blending bioplastics into their current drink bottles and plans to produce bioplastic-only bottles in the future (The Coca-Cola Company 2017). Such examples provide strong evidence for a growing market and uptake of bioplastic-based products and their potential importance in the future polymer industry.

5.1.2 Bioplastics and Polyhydroxyalkanoates

Polyhydroxyalkanoates are a promising bio-derived, biocompatible, biodegradable, and chemically diverse polyester material (Serafim et al. 2008). They are synthesized naturally in the prokaryotic microbial cells under conditions that induce a need for carbon, energy, or reducing-power storage (Salehizadeh and van Loosdrecht 2004; Castilho et al. 2009). In addition, they can also be produced by plants. However, due to the low yield in plants of less than 10% of dry weight polyhydroxyalkanoates (Verlinden et al. 2007) and limited polyhydroxyalkanoates monomer types produced (Gumel et al. 2013), bacteria are the more promising production route.

In bacteria, polyhydroxyalkanoates are produced in the process of carbon assimilation under unbalanced conditions as an intracellular storage product (Bugnicourt et al. 2014) and can be consumed by the bacteria as an alternative carbon source in conditions of famine (Kourmentza et al. 2017). Over 90 genera of microbial species are known to produce polyhydroxyalkanoates and are found in a wide variety of environments (Castilho et al. 2009). Bacteria store polyhydroxyalkanoates inside the cell in a polymerized form (Saharan et al. 2014), and their production can reach a yield of up to 90% of dry weight polyhydroxyalkanoates in some special cases (Verlinden et al. 2007). They are found under different types and shapes and are produced by different strains of bacteria and some archaea (Koller et al. 2011; Tan et al. 2014). In fact, more than 150 different monomer units have been linked as constituents of polyhydroxyalkanoates (Castilho et al. 2009). Hydroxybutyrate, hydroxyvalerate, hydroxyhexanoate (Green et al. 2002), hydroxypentenoate, hydroxyoctanoate, hydroxypropionate (Kourmentza et al. 2017), hydroxydecanoate (Hokamura et al. 2017), and hydroxyheptanoate (Green et al. 2002) are few of the many monomer units that can compose polyhydroxyalkanoates in addition to many other hydroxyalkanoic acids and even some mercaptoalkanoic acids (Visakh 2014). The general structure of polyhydroxyalkanoates is shown in Fig. 5.2. Usually, a polyhydroxyalkanoate molecule is composed of 600 to 35,000 monomer units (Tan et al. 2014). When various monomer types are incorporated into the chain, the properties of the polymer change including a decrease in the melting temperature, especially with the incorporation of more hydroxyvalerate units into hydroxybutyrate units (Fradinho et al. 2014). Additionally, it was discovered that polyhydroxyalkanoates with 4-hydroxybutyrate have higher flexibility than the 3-hydroxybutyrate (Możejko-Ciesielska and Kiewisz 2016).

Fig. 5.2 Basic repeating structure of polyhydroxyalkanoates

Types of Polyhydroxyalkanoates

The first discovered polyhydroxyalkanoate was poly(3-hydroxybutyrate) back in 1926 from *Bacillus megaterium* as it is an abundant homopolymer (Castilho et al. 2009). Since then, a wide range of homo- and co-polymers have been discovered. Polyhydroxyalkanoates are frequently classified into three groups based on the number of carbon atoms within the monomer. Chains with three to five carbon atoms are classified as short chain length (scl) and are more common, while chains with six to 14 carbons are medium chain length (mcl), for which *Pseudomonas* are prominent producers (Lutke-Eversloh and Steinbuchel 2004). Chains with more than 14 carbons are classified as long-chain-length (lcl) polyhydroxyalkanoates (Troschl et al. 2017).

The two most common types of polyhydroxyalkanoates are polyhydroxybutyrate and poly(hydroxyvalerate) and are both classified as scl-polyhydroxyalkanoates. Their copolymers such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate) are also scl-polyhydroxyalkanoates. The most common mcl-polyhydroxyalkanoate is poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). In general, many factors are involved in the development of the structural composition of polyhydroxyalkanoates including the substrate, strains of bacteria and environment properties, although both wild-type and recombinant bacteria have the ability to produce scl- and mcl-polyhydroxyalkanoates (Tan et al. 2014; Dietrich et al. 2017).

Polyhydroxyalkanoates Properties

The combination of high functionality with the low environmental impact that polyhydroxyalkanoates display makes them promising candidates for sustainable polymer production (Dietrich et al. 2017). Due to the diversity of polyhydroxyalkanoates, its properties and subsequent uses are various and depend on factors such as type(s) of monomers and producing strains of bacteria among others (Castilho et al. 2009). For instance, scl-polyhydroxyalkanoates are usually employed in one-time-use plastics for food packaging, while the mcl-polyhydroxyalkanoates are suitable for applications in the medical field such as sutures and implants due to their characterization as elastomers (Kourmentza et al. 2017). Polyhydroxybutyrate is known for its low oxygen permeability, moisture and ultraviolet light resistance, optical purity, and similar thermoplastic properties to polyethylene and polypropylene (Salehizadeh

and van Loosdrecht 2004; Verlinden et al. 2007; Muralidharan et al. 2013), but has inferior mechanical properties than petroleum-based plastics as it is brittle and has a high crystallinity degree (Castilho et al. 2009; Visakh 2014). Nevertheless, polyhydroxyalkanoates have many advantages such as their tunable mechanical and physical properties as well as a low environmental impact (Dietrich et al. 2017). Moreover, copolymers of polyhydroxybutyrate and polyhydroxyvalerate are known to be less permeable to oxygen than polyethylene and polypropylene (Salehizadeh and van Loosdrecht 2004). In the food packaging field, this property is important as it reduces the need for antioxidants.

The mechanical properties of polyhydroxyalkanoates vary significantly and can be tuned based on the type of substrate, bacteria, and fermentation conditions used to derive varying homo- and co-monomer units (Dietrich et al. 2017). Moreover, the mechanical properties of a bioplastic are strongly connected to the average molecular weight of the polymer (Salehizadeh and van Loosdrecht 2004). The homopolymer polyhydroxybutyrate is highly crystalline (70–80% crystallinity), meaning it has a low impact strength and low resistance to brittle failure (Salehizadeh and van Loosdrecht 2004; Bugnicourt et al. 2014). It was also reported that polyhydroxybutyrate displays a high tensile strength with a value between 30 and 43 MPa (Verlinden et al. 2007; Bugnicourt et al. 2014). On the other hand, polyhydroxybutyrate-co-valerate has better mechanical properties than polyhydroxybutyrate due to an increase in flexibility, toughness, and strength influenced by the addition of hydroxyvalerate units in the polyhydroxyalkanoates polymer (Salehizadeh and van Loosdrecht 2004). It was also discovered that the molar fraction of hydroxybutyrate units increased the crystallinity and made the material less ductile because of the short chain length of the polyhydroxybutyrate monomers (Li et al. 2016). Glass transition temperature is found to be ranging from −52 °C to 4 °C. The melting temperature was recorded around 175–177 °C, while the thermodegradation temperature is in the range of 227 °C and 256 °C (Bugnicourt et al. 2014; Tan et al. 2014). Pure polyhydroxybutyrate was found to have a very low resistance to temperature as it can decompose at room temperature (Bugnicourt et al. 2014). It was also discovered that the existence of valerate in the polyhydroxyalkanoates chain increases their thermal stability (Verlinden et al. 2007). Table 5.1 summarizes some mechanical properties, temperature transition points, and crystallinity values of various types of polyhydroxyalkanoates.

Polyhydroxyalkanoates have a molar mass that varies according to the producing organism, growth environment, and extraction method employed (Bugnicourt et al. 2014). Usually, it is high in the range of 100–1000 kDa. However, the highest molar mass achieved by polyhydroxyalkanoates was about 20,000 kDa and reported to be produced by recombinant *Escherichia coli* (Castilho et al. 2009). However, the chain length has a more significant role and affects many properties of the polyhydroxyalkanoates including melting temperature, glass transition temperature, hydrophobicity, crystallinity, and mechanical strength (Castilho et al. 2009; Li et al. 2016). While the scl-polyhydroxyalkanoates are highly crystalline and display high melting temperature, the mcl-polyhydroxyalkanoates have low melting temperatures and low crystallinity. In general, polyhydroxyalkanoates display low

Table 5.1 Mechanical and thermal properties of various polyhydroxyalkanoates types. Scl, short chain length; mcl, medium chain length

	scl-polyhydroxyalkanoates (Możejko-Ciesielska and Kiewisz 2016)	Polyhydroxybutyrate (Verlinden et al. 2007; Bugnicourt et al. 2014)	Blends of mcl-polyhydroxyalkanoates (Możejko-Ciesielska and Kiewisz 2016; Wang et al. 2014)
Extension to break (%)	40	5	3–680
Tensile strength (MPa)	5	30–43	17–690
Young's modulus (GPa)	3.5	1–2	0.7–2.9
Glass transition temperature (°C)	4	−1	−43–−25
Melting temperature (°C)	179	177	39–170
Thermal degradation temperature (°C)	NA	180	210–215
Crystallinity (%)	55–80	70–80	25–60

degradation temperatures (Visakh 2014), which can be overcome by the incorporation of varied monomer units into the chain.

In order to improve the performance of polyhydroxyalkanoates and lower their costs, various types of polyhydroxyalkanoates can be blended together or with other types of biodegradable plastics such as polylactic acid and polycaprolactone. The blending methodology enables the addition of new functionalities into the polymer and limits its drawbacks, resulting in higher mechanical strength and improved surface features and amphiphilicity (Li et al. 2016; Visakh 2014). Additionally, blends of polyhydroxybutyrate and polyhydroxybutyrate-co-3-hydroxyhexanoate prepared by Yang et al. revealed a lower degree of crystallization (Yang et al. 2002), which was later confirmed by the work of Lim et al. that prepared a blend of polyhydroxybutyrate-co-3-hydroxyhexanoate and polycaprolactone (Lim et al. 2013). Another research group mixed polyhydroxybutyrate with starch, which resulted in materials approved for usage in the medical field. They prepared a blend of 30:70 ratio of starch to polyhydroxybutyrate, and characterization results revealed a higher tensile strength compared to a pure polyhydroxybutyrate polymer (Godbole et al. 2003). Experiments where cellulose derivatives were mixed with polyhydroxybutyrate revealed that as the concentration of polyhydroxybutyrate decreased

in the polymer, its glass transition temperature increased, which gives a larger temperature range of operation in the rigid state, but limits their temperature range for processing and use in a rubbery and flexible state (Zhang et al. 1997).

Once the polyhydroxyalkanoates are disposed into the environment, they will easily degrade into carbon dioxide and water aerobically by microorganisms (Chee et al. 2010). The period of degradation will vary according to the environment. According to the American Society for Testing and Materials (ASTM) standards, polyhydroxyalkanoates are compostable and biodegradable materials in marine environments. If disposed into anaerobic sewage, it will take 6–8 months to degrade, while it will take a few years to degrade in seawater. The presence of UV light increases the rate of polyhydroxyalkanoates degradation (Verlinden et al. 2007). In natural aerobic environments, polyhydroxyalkanoates usually degrade after 60 to 75 weeks (Brandl et al. 1990).

5.2 Polyhydroxyalkanoates Production

5.2.1 Current Commercial Products

Polyhydroxyalkanoates production is already in progress, although it is very limited comprising only 2.4% of global bioplastic production in 2017 (European Bioplastics 2017). The lack of further implementation of polyhydroxyalkanoates production on a larger scale is due to the high production costs compared to the petrochemical industry alternatives (Tan et al. 2014). Currently, the cost of polypropylene and polyethylene ranges from US$0.6 to 0.87 for one pound, while the polyhydroxyalkanoates prices is within US$2.25–2.75 per pound (Kourmentza et al. 2017). Current industrial polyhydroxyalkanoates production relies on pure culture fermentation, which increases the overall costs due to the need of sterilization (Fradinho et al. 2013b). Moreover, fermentation processes are costly due their low yield per substrate, which typically lie in the range of 18–50% (Salehizadeh and van Loosdrecht 2004). Additional challenges include control of polyhydroxyalkanoates structure and properties (Dietrich et al. 2017).

There are four major components to the polyhydroxyalkanoates high costs: cost of substrate used, productivity, yield per substrate, and the recovery method (Dietrich et al. 2017). Choosing a suitable substrate is an important factor in the production of polyhydroxyalkanoates as it affects significantly the costs of production and the potential polyhydroxyalkanoates produced. In fact, over 40% of the polyhydroxyalkanoates production expenses may be related to the raw materials, and about 70% of this cost is related to the carbon source (Salehizadeh and van Loosdrecht 2004). There are substrates that are rich in carbon and nutrients and are associated with growth. There are also substrates rich in carbon only with no nutrients, and those are not associated with growth (Kourmentza et al. 2017). The most widely used substrates are glucose, fructose, and fatty acids (Dietrich et al. 2017).

However, in most cases, the most inexpensive raw material options are from wastes and by-products making organic-rich wastewaters a particularly attractive feedstock option (Castilho et al. 2009).

Sugars as a carbon source produce a low yield of polyhydroxyalkanoates. For example, the highest yield of 0.4 g of polyhydroxyalkanoates per g sugar was produced using glucose as a carbon source (Chee et al. 2010). Experiments conducted on glucose as the only carbon source in a two-step fermenter accumulated a polyhydroxyalkanoates content of 17.2% of cell dry weight (Gonzalez-Garcia et al. 2008). Agroindustrial wastes such as cheese whey, beet molasses, and plant oils are cheap substrates for the production of polyhydroxyalkanoates. Plant oils are usually cheaper than sugar sources and are great carbon sources for the production of polyhydroxyalkanoates and give a high yield of polyhydroxyalkanoates around 0.6 to 0.8 g of polyhydroxyalkanoates per g of oil used (Chee et al. 2010). Moreover, cheese whey is among the cheapest polyhydroxyalkanoates carbon sources, costing $0.22/kg, and produces a polyhydroxyalkanoates yield of 0.33 g-polyhydroxybutyrate/g. In comparison, petrochemical polypropylene substrate costs are around $0.185/kg (Salehizadeh and van Loosdrecht 2004). Using cheap agroindustrial wastes as a substrate will result in a decrease of costs. However, to be truly cost competitive, there is a need to use even lower cost, or free, substrates. Wastewaters provide an opportunity in this regard as not only is the substrate free of cost, but cost offsetting against their treatment can be realized.

The next major cost influencer is productivity, which is related to substrate. In experiments conducted by Lee et al., it was revealed that the cost of polyhydroxybutyrate production by *Azohydromonas lata* (previously known as *Alcaligenes latus*) decreased as the polyhydroxybutyrate production rate increased. The costs decreased from $4–5/kg polyhydroxybutyrate to $2.6/kg-polyhydroxybutyrate as the polyhydroxybutyrate productivity increased from 1.98 g/L/h to 3.2 g/L/h (Salehizadeh and van Loosdrecht 2004; Lee and Choi 1998).

The polyhydroxyalkanoates content of the microbial biomass also impacts the efficiency of the recovery process. For instance, a polyhydroxybutyrate content of 50% results in a high recovery cost in the order of $4.8/kg-polyhydroxybutyrate, while obtaining a high polyhydroxybutyrate content of 88% would reduce the recovery cost to only $0.92/kg-polyhydroxybutyrate. This is related to the large use of digesting agents for breaking the cell walls and the increased cost of waste disposal (Salehizadeh and van Loosdrecht 2004).

In an effort to reduce the production costs of polyhydroxyalkanoates, a focus on identification and genetic engineering of bacterial strains and improvements in efficiency of fermentation/recovery processes have been undertaken (Salehizadeh and van Loosdrecht 2004). The price of industrial polyhydroxyalkanoates was 15–17 times the price of petrochemical polymers back in 2004. Thanks to further research, the price has reduced to three times that of petroleum-based polymers (Dietrich et al. 2017). However, the cost of production is still a challenge that needs to be overcome, and in general the consumer is not ready to pay a significantly higher cost for a material with biodegradable properties. Further research on the effects of

low-cost mixed cultures and high productivity recombinant microbial strains could help decrease the costs further (Visakh 2014).

There are many factors involved in the accumulation of polyhydroxyalkanoates that could be modified and improved to increase the production. Earlier research focused on the feeding of the system and alternating aerobic and anaerobic environments (Kourmentza et al. 2017). Recently, new strategies are under development to improve and combine polyhydroxyalkanoate-producing strains with advanced fermentation designs that could lower the costs of polyhydroxyalkanoates production and simultaneously reduce the waste in the environment. Another strategy would be to develop functional strains with technology that have the ability to control the structures of polyhydroxyalkanoates monomers developed (Wang et al. 2014). In fact, many variables influence the production of polyhydroxyalkanoates including the aeration, feeding process, electron donor/acceptor availability, carbon and energy sources, temperature and pH of the environment, and nutrient availability (Kourmentza et al. 2017; Montiel-Jarillo et al. 2017). However, the easiest approach to reduce the costs considerably is to employ cheap, renewable carbon substrates readily available in industrial wastewaters, particularly agroindustrial, with the use of low-maintenance mixed cultures for combined polyhydroxyalkanoates production and wastewater treatment.

5.2.2 Biological Aspects of Production

Many different types of bacteria produce polyhydroxyalkanoates as part of their usual metabolism, including archaea and Gram-positive and Gram-negative bacteria (Chee et al. 2010). Polyhydroxyalkanoates support the survival of bacteria in case of nutrient-scarce or fluctuating organic conditions, being accumulated under these conditions within the cell for later use as a carbon and energy source (Fradinho et al. 2016).

There are three main pathways for the production of polyhydroxyalkanoates by microorganisms. The first pathway depends on the carbon substrate used and relies on three enzymes: ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC) (Możejko-Ciesielska and Kiewisz 2016). This pathway usually results in scl-polyhydroxyalkanoates due to the involvement of short-chain volatile fatty acids such as acetic acids, propionic acid, butyric acid, and valeric acid and is summarized in Fig. 5.3 (Serafim et al. 2008). The biosynthesis of polyhydroxyalkanoates, or polyhydroxybutyrate specifically, from sugars begins by taking up acetate as a substrate and converting it to acetyl-CoA. Then two molecules of acetyl-CoA are condensed into acetoacetyl-CoA, which is then reduced to 3-hydroxybutyl-CoA and finally polymerized into polyhydroxybutyrate (Salehizadeh and van Loosdrecht 2004; Bugnicourt et al. 2014). Propionate can result in three different types of polyhydroxyalkanoates depending on the precursor. The polymer poly-3-hydroxy-2-methylvalerate is a result of two molecules of propionyl-CoA. Either polyhydroxyvalerate or poly-3-hydroxy-2-methylbutyrate

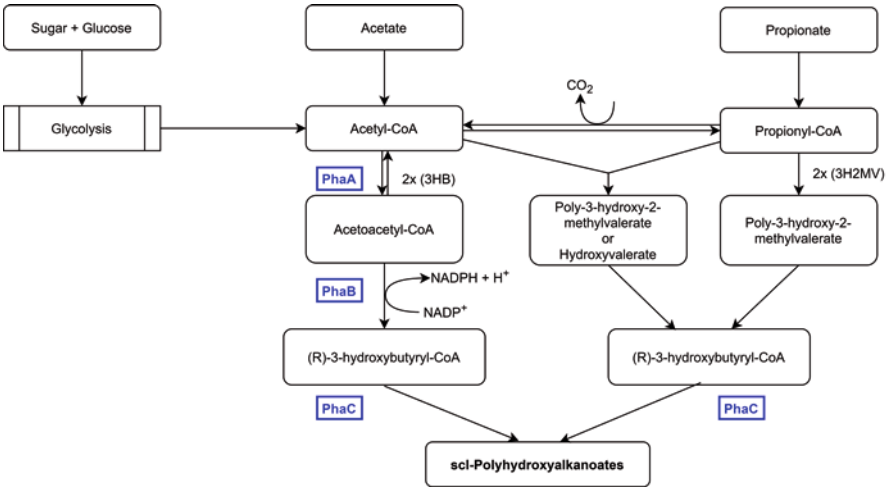

Fig. 5.3 Pathway I for short-chain-length polyhydroxyalkanoates production (Modified after Serafim et al. 2008). Scl, short chain length

can be the result of the combination of one molecule of propionyl-CoA and one molecule of acetyl-CoA (Serafim et al. 2008). This production route is usually known as pathway I for polyhydroxyalkanoates production.

The two other polyhydroxyalkanoates pathways are associated with the accumulation of mcl-polyhydroxyalkanoates because longer-chain volatile fatty acids are implicated in the process. In the second known pathway, the carbon source, typically fatty acids, is oxidized to 3-hydroxyacyl-CoA as summarized in Fig. 5.4. This reaction is conducted by enoyl-CoA hydratase (PhaJ) and PhaC enzymes. The conversion of fatty acids to polyhydroxyalkanoates involves a few steps and results in high yields (Kourmentza et al. 2017). The last known pathway is a chain elongation from simple carbons, and a regulatory protein (PhaG) and PhaC are the main enzymes, as explained in Fig. 5.5 (Możejko-Ciesielska and Kiewisz 2016). Additionally, random copolymers of polyhydroxyalkanoates are usually produced when mixed substrates are employed, which occurs during pathway II and III (Verlinden et al. 2007).

Genetic engineering is also used to modify the pathway of polyhydroxyalkanoates production and to increase the yield and simultaneously decrease the expenses (Lutke-Eversloh and Steinbuchel 2004). Experiments conducted on *Escherichia coli* revealed that a production of 90% of polyhydroxybutyrate was reached via this method, where the PhaC enzyme was added to the *Escherichia coli* from *Cupriavidus necator* (Możejko-Ciesielska and Kiewisz 2016; Koller et al. 2017). While the results of genetically modified bacteria are great, the costs are high for their development and for maintaining the pure culture during production, making them typically not suitable for wastewater treatment applications.

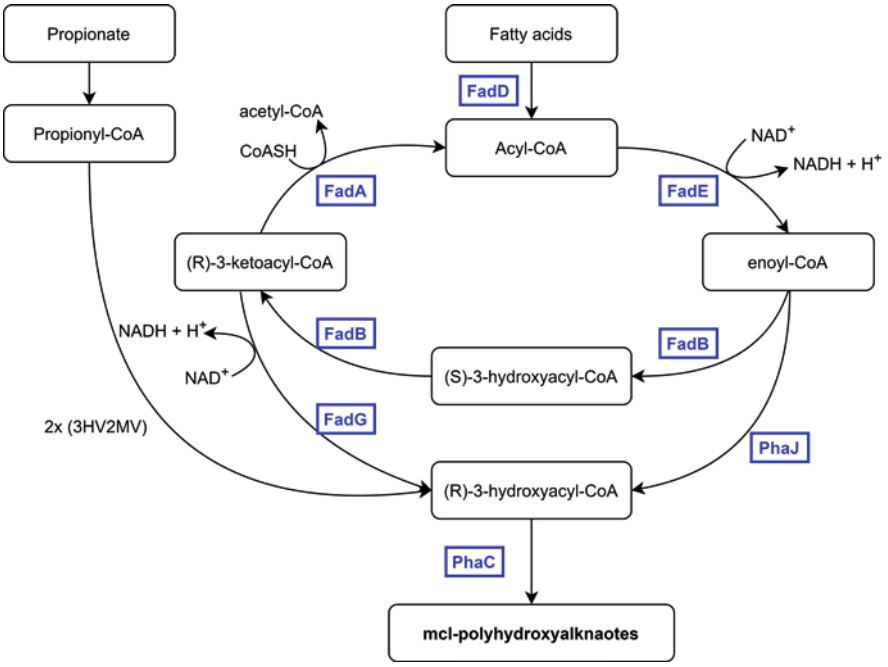

Fig. 5.4 Pathway II for medium-chain-length polyhydroxyalkanoates production (Modified after Serafim et al. 2008). Mcl, medium chain length

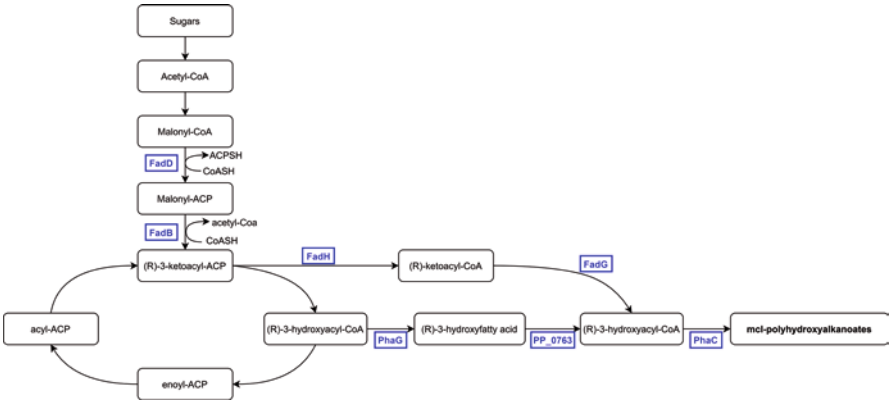

Fig. 5.5 Pathway III for mcl-polyhydroxyalkanoates production (Modified after Serafim et al. 2008). Mcl, medium chain length

5.3 Industrial Wastewater Importance

Industrial effluents are highly varied from industry to industry and can present a major pollution threat to the environment if not sufficiently treated. While the production of polyhydroxyalkanoates by microbial mixed cultures treating wastewaters was first discovered in municipal wastewater plants, in 1974 (Serafim et al. 2008), many industrial wastewaters may be more suitable due to their typically rich, often soluble, organic content and limited nutrients, which allows the possibility to exploit typical polyhydroxyalkanoates accumulation mechanisms (Saharan et al. 2014). The imbalance of nutrients within industrial waters makes them naturally suitable to drive organic conversion toward polyhydroxyalkanoates rather than cell biomass (Valentino et al. 2016). Organics present in industrial wastewater are typically in high concentration helping to promote high rates of polyhydroxyalkanoates production and may consist of a limited range of organic compounds that can direct selective polyhydroxyalkanoates production.

Many food and agricultural industries produce carbon-rich substrates suitable for polyhydroxyalkanoates production. The cheese and dairy industry are among the biggest producers with protein-rich wastes, followed by the potato industry that produces waste rich with starch (Koller et al. 2017). Olive oil wastewater is an abundant source of carbon-rich feedstocks and is found in many areas around the globe, especially in the Mediterranean region. Olive mill wastewater was tested as a carbon source for the production of polyhydroxyalkanoates and accumulated 43% polyhydroxyalkanoates of cell dry weight in a one-step batch (Beccari et al. 2009; Kourmentza et al. 2009). The wine industry, also, has potential for the production of polyhydroxyalkanoates. One research group used grape pomace from wine production for the accumulation of polyhydroxyalkanoates. They employed a two-stage fermenter filled with *Pseudomonas putida*. The first stage consisted of the sugar extracted from the pomace for growth, while the second stage relied on a mixture of octatonic and undecenoic acids for the polyhydroxyalkanoates accumulation phase. The experiment was conducted in nitrogen-limiting conditions in a fed-batch mode in a 100 L fermenter. Under these conditions, the wine waste was able to produce a 41.1% of poly(3-hydroxyoctanoate-co-3-hydroxy-10-undecenoate) (Follonier et al. 2015).

In some cases, the wastewater from industries was tested following a pretreatment step of fermentation in order to convert sugars to fatty acids and increase the polyhydroxyalkanoates yield (Kourmentza et al. 2017). Wastewater of tomato production was used for polyhydroxyalkanoates production and accumulated 30–39% of cell dry weight of polyhydroxyalkanoates (Bengtsson et al. 2017). Wastewater from a milk and ice-cream processing plant produced a polyhydroxyalkanoates content of 42.5% of cell dry weight (Chakravarty et al. 2010), while that from a confectionary bar plant accumulated a higher polyhydroxyalkanoates value of 70–76% of cell dry weight (Tamis et al. 2014).

5.4 Factors Influencing Polyhydroxyalkanoates Production, Accumulation, and Composition

5.4.1 Strains of Bacteria

There are many microorganisms with the ability of producing polyhydroxyalkanoates from carbon sources. Many groups of naturally occurring prokaryotes, such as bacteria and archaea, can decompose and consume polyhydroxyalkanoates for their survival and growth (Dietrich et al. 2017). There are more than 300 microorganisms with ability to produce polyhydroxyalkanoates (Choi and Lee 1999). However, the main issue is the selection of bacterial strains with high polyhydroxyalkanoates production rates and accumulation capabilities. In the past, polyhydroxyalkanoates have been industrially produced by pure cultures including *Azotobacter vinelandii, Azohydromonas lata*, and *Pseudomonas oleovorans* at high concentrations (Salehizadeh and van Loosdrecht 2004; Choi and Lee 1999). The most widely used wild-type strain for scl-polyhydroxyalkanoates is *Cupriavidus necator* due to its well-known metabolism, its ability to grow at high cell density (100–200 g/L), and its high cellular polyhydroxyalkanoates content (75–80%) (Dietrich et al. 2017). For mcl-polyhydroxyalkanoates production, the most commonly employed bacteria is *Aeromonas hydrophila* (Dietrich et al. 2017; Choi and Lee 1999). *Escherichia coli* bacteria is the most widely used recombinant bacteria in the industry because of its easy genetic manipulation, fast growth, and ability to use cheap carbon sources (Dietrich et al. 2017).

Pseudomonas have a wide catabolic versatility, which enables them to reduce diverse carbon sources such as toluene, benzene, and ethylbenzene (Kourmentza et al. 2017). They also have the ability to grow in soils polluted with petroleum or petroleum-based hydrocarbons and therefore have promise for polyhydroxyalkanoates production using industrial wastewaters including petrochemical, oil, and gas industry wastewaters (Goudarztalejerdi et al. 2015). *Pseudomonas* were able to produce a high content of polyhydroxyalkanoates at 65.8% from decanoic acid as the sole carbon source, while benzene and toluene produced 19.1% and 58.9% under a continuous feeding condition, respectively (Kourmentza et al. 2017).

Among the microorganisms capable of anaerobic storage of carbon sources are the well-known polyphosphate-accumulating organisms and glycogen-accumulating organisms. Polyphosphate-accumulating organisms are one of the well-known polyhydroxyalkanoates producers, not due to their high production rates or yields but due to their competitive advantage associated with their ability to utilize energy stored as polyphosphate to convert and store external substrate in the form of polyhydroxyalkanoates when no electron acceptor for energy generation is available (Salehizadeh and van Loosdrecht 2004). This metabolism is widely exploited for phosphorus removal from wastewaters allowing easy enrichment in mixed cultures. Glycogen-accumulating organisms are a natural competitor to polyphosphate-accumulating organisms and produce polyhydroxyalkanoates from fermentation products and from stored glycogen (Salehizadeh and van Loosdrecht 2004). The

energy released from glycolysis is used to incorporate intracellular fermentation products in the form of polyhydroxybutyrate. Both polyphosphate-accumulating organisms and glycogen-accumulating organisms thrive in a cyclic environment where the substrate is periodically present when a preferred electron acceptor is absent (Salehizadeh and van Loosdrecht 2004). However, their polyhydroxyalkanoates production is typically limited to only 20–30% cell dry weight (Serafim et al. 2008), though values up to 60% have been reported (Albuquerque et al. 2007).

Archaea, which are typically extremophiles, are another group of microorganisms with the ability to produce polyhydroxyalkanoates. Known archaeal polyhydroxyalkanoates producers are all halophiles, requiring high salinity environments to support their growth (Saharan et al. 2014). The advantage of producing polyhydroxyalkanoates with halophiles is the reduction and elimination of contamination from unwanted species under open or mixed culture cultivation (Kourmentza et al. 2017). The first case of archaea-producing polyhydroxyalkanoates was recorded in the Dead Sea in 1970. Since then, many experiments were conducted where archaea produced up to 65% polyhydroxyalkanoates of cell dry weight under nutrient-limiting conditions (Saharan et al. 2014). Currently, the best archaea for polyhydroxyalkanoates production is *Haloferax mediterranei*, frequently found in the coast of Spain (Kourmentza et al. 2017). Studies conducted on this type of bacteria revealed that low-cost feedstocks result in a high polyhydroxyalkanoates content. In an experiment, pretreated vinasse was used as the carbon source and produced 66–70% of polyhydroxyalkanoates (Bhattacharyya et al. 2014).

Pure cultures have historically been used to produce polyhydroxyalkanoates, but their use is still limited by the high costs of maintaining cultures through sterilization in special equipment (Kourmentza et al. 2017). In contrast, microbial mixed cultures are less costly to maintain, particularly with feedstocks already rich in microorganisms (Kourmentza et al. 2017; Valentino et al. 2016). Microbial mixed cultures reduce fermentation equipment expenses and typically require less process control (Serafim et al. 2008). Additionally, microbial mixed cultures can be manipulated in order to produce specific polyhydroxyalkanoate polymers by varying the carbon source composition as well as the feeding cycle (Gumel et al. 2013). These factors make mixed cultures an excellent candidate to couple with wastewater treatment where the feedstocks are effectively free or even better can be offset against costs typically associated with treatment.

Activated sludge is a generic term for flocculated microbial mixed cultures frequently used in wastewater treatment. Dominant polyhydroxyalkanoate-producing bacteria found in wastewaters include *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Chromobacterium* (Koller et al. 2011), *Plasticicumulans* (Jiang et al. 2011b, c), *Azoarcus, Paracoccus*, and *Thauera* (Albuquerque et al. 2012). These cultures have the ability to store polyhydroxyalkanoates as carbon and energy storage material. They can accumulate polyhydroxyalkanoates under conditions of irregular nutrient availability due to an intermittent feeding regime and presence of an electron acceptor (Serafim et al. 2008). The microorganisms present are able to adapt to rapidly changing conditions of nutrient availability and continuous changes in substrate (Salehizadeh and van Loosdrecht 2004). Beccari et al. reported that with a microbial

mixed culture fed with acetate-limited conditions and intermittent feeding that polyhydroxybutyrate accumulated up to 90% of cell dry weight (Beccari et al. 1998). Fradinho et al. explored the effects of malate, citrate, lactate, acetate, propionate, and butyrate on polyhydroxyalkanoates accumulation in phototrophic microbial mixed cultures under continuous light. While microbial mixed culture fed with malate, citrate, and lactate did not accumulate polyhydroxyalkanoates, cultures fed with acetate, propionate, and butyrate were able to store polyhydroxyalkanoates (Fradinho et al. 2014). While the mixed culture reduces polyhydroxyalkanoates production costs significantly, it produces a low cell density compared to the pure cultures, which can make subsequent extraction steps more costly (Kourmentza et al. 2017).

5.4.2 Redox Environment

Reports on the impact of redox environment on polyhydroxyalkanoates production are mixed. In the study of Gumel et al., it was observed that aerobic environments resulted in a lower accumulation of polyhydroxybutyrate compared to anaerobic ones, which is related to the shift of cellular activities caused by the availability of oxygen (Gumel et al. 2013). The cells shift to produce energy-intensive protein and glycogen because of the availability of NAD+ and ATP under aerobic conditions and halt the production of polyhydroxyalkanoates due to the energy-rich nature of aerobic oxidation reactions (Saharan et al. 2014; Third et al. 2002). In a sequencing batch reactor experiment with acetate as the carbon source, it was revealed that at lower dissolved oxygen conditions, the acetate conversion rate was low, but the majority of the substrate was converted to polyhydroxybutyrate compared with higher dissolved oxygen conditions. The low ATP available was used for the transportation of acetate into the cells (Third et al. 2002). Another experiment testing the impact of the redox environment on polyhydroxyalkanoates production revealed that microaerobic environments produced higher polyhydroxyalkanoates content up to 56% of cell dry weight compared to only 34% of cell dry weight under aerobic conditions (Amulya et al. 2016). Similar findings in a separate study showed cell dry weight storage can be increased from 20% and 33% under anaerobic and aerobic conditions, respectively, to 62% in a microaerophilic-aerobic sludge process (Satoh et al. 1998).

In contrast, some experiments have found that polyhydroxyalkanoates production rates using aerobic systems are higher. For instance, a study using a sequencing batch reactor found polyhydroxyalkanoates conversion yields of 0.7–0.9 C-mol/C-mol (Filipe et al. 2001) compared to anoxic conditions 0.4–0.5 C-mol/C-mol (Beun et al. 2002). Similarly, Wang et al. evaluated the effect of dissolved oxygen on the substrate competition and polyhydroxyalkanoates production in microbial mixed cultures with mixed volatile fatty acids as carbon source. The change of dissolved oxygen levels in the environment affected the polyhydroxyalkanoates storage capability and yield, where a high dissolved oxygen level resulted in high

polyhydroxyalkanoates accumulation rates (Wang et al. 2017). The effects of dissolved oxygen were different for different volatile fatty acid substrates.

The role of alternating anaerobic and aerobic conditions to promote polyhydroxyalkanoates storage is well known due to its widespread implementation to drive biological phosphorus removal. In this process, carbon is stored in an anaerobic period when it is readily available and then used in an aerobic period to drive more efficient cell growth, which in the case of polyphosphate-accumulating organisms is associated with a replenishment of polyphosphates that can be used as an energy source in the anaerobic phase to convert volatile fatty acids as polyhydroxyalkanoates. This approach is useful in wastewaters rich in nutrients (Saharan et al. 2014).

Alternating redox conditions have also been shown to alter the monomer composition of the polyhydroxyalkanoates produced. Work conducted by Bengtsson et al. on a carbon source from a paper mill waste under anaerobic conditions produced polyhydroxyalkanoates containing hydroxybutyrate, hydroxyvalerate, and poly-3-hydroxy-2-methylvalerate monomers at 6%, 47%, and 47%, respectively, with glycogen-accumulating organisms as the main microorganism. The same research group using a similar wastewater under aerobic followed by anaerobic conditions achieved polyhydroxyalkanoates with similar monomers produced but at different concentrations with a ratio of 33%, 51%, and 16%, respectively (Bengtsson et al. 2008). In a separate study conducted by Dai et al. to evaluate the impact of acetate as a substrate on the polyhydroxyalkanoates composition under aerobic conditions, the polyhydroxyalkanoates produced contained 93% of hydroxybutyrate monomers and 7% of hydroxyvalerate monomers. However, when the feeding mode was changed to an aerobic/anaerobic one, the production of hydroxyvalerate monomers increased to 30%, while the hydroxybutyrate monomers' production dropped to 70% (Dai et al. 2007). Furthermore, experiments conducted by Amulya et al. revealed that under microaerophilic environments and glucose as a substrate, the content of hydroxyvalerate monomers increased to 21% compared to 8% under aerobic conditions (Amulya et al. 2016).

5.4.3 Effects of Carbon Source and Raw Materials

Effect on Polyhydroxyalkanoates Composition

Many factors influence the final polyhydroxyalkanoates composition including the type of substrate employed. Diverse monomer production is linked to substrates containing a variety of volatile fatty acids (Serafim et al. 2008), making complex industrial wastes an interesting feedstock where fermentation conditions could be used to control the final polyhydroxyalkanoates product. Additionally, volatile fatty acids with odd vs even chain lengths were also found to favor the production of hydroxybutyrate or hydroxyvalerate (Morgan-Sagastume et al. 2015). Acetate is one of the most popular carbon sources for polyhydroxyalkanoates production and

also one of the most prevalent following fermentation of complex feedstocks. If used as a carbon source alone, it results in higher polyhydroxybutyrate content and in some cases pure polyhydroxybutyrate. Butyrate alone was found to produce only polyhydroxybutyrate, while the presence of propionate results in a higher polyhydroxyvalerate content (Saharan et al. 2014). In an experiment conducted by Bengtsson et al., the impact of acetate, propionate, butyrate, and valerate on glycogen-accumulating organisms for polyhydroxyalkanoates production was investigated under the same conditions. They found that acetate resulted in the highest hydroxybutyrate/hydroxyvalerate ratio at 89:11, followed closely by a pure butyrate substrate at a ratio of 83:7 hydroxybutyrate/hydroxyvalerate, along with new monomers. A pure valerate carbon source produced the highest hydroxyvalerate content at a ratio of 12:78 hydroxybutyrate/hydroxyvalerate, followed by propionate at a ratio of 12:63 hydroxybutyrate/hydroxyvalerate (Bengtsson et al. 2010). Yu et al. explored the impact of various concentrations of butyrate and valerate using *Cupriavidus metallidurans* (previously known as *Alcaligenes eutrophus*) on their ability to accumulate various types of monomers. The research revealed that 100% butyrate substrate resulted in polyhydroxybutyrate monomers only, while 100% valerate substrate resulted in 46:54 hydroxybutyrate/hydroxyvalerate (Yu et al. 1998). Polyhydroxyalkanoates experiments conducted by Takabatake et al. confirmed the relationship between acetate and propionate substrates and their impact on the hydroxybutyrate/hydroxyvalerate ratio, observing a 97:3 hydroxybutyrate/hydroxyvalerate monomer ratio with acetate feed and 16:84 hydroxybutyrate/hydroxyvalerate ratio with propionate as substrate (Takabatake et al. 2000). Mixed cultures fed with mixed carbon sources result in more diverse monomer production. Lemos et al. produced a polyhydroxyalkanoates polymer with hydroxybutyrate/hydroxyvalerate/H2MV at 6:58:24 ratio using microbial mixed culture with a mixture of acetate and propionate (Lemos et al. 2006). Jiang et al. conducted experiments on microbial mixed cultures with acetate, propionate, and an acetate-propionate mixture. While there was little change to the dominating microorganism as the substrate changed, they discovered a relationship between the concentrations of acetate and propionate and the ratio of hydroxybutyrate/hydroxyvalerate monomers produced (Jiang et al. 2011a). Yu et al. used malt waste on *Cupriavidus metallidurans*, *Azohydromonas lata*, and an activated sludge. While the pure cultures resulted in polyhydroxybutyrate monomers only, the activated sludge produced 92:8 hydroxybutyrate/hydroxyvalerate (Yu et al. 1998).

Pure culture studies highlight the influence of substrate on both polyhydroxyalkanoates production and polyhydroxyalkanoates monomer composition. *Cupriavidus necator* is a widely utilized organism for polyhydroxyalkanoates production as it has displayed the interesting ability to not only produce polyhydroxyalkanoates under heterotrophic growth but also to produce polyhydroxyalkanoates under autotrophic metabolism with hydrogen. *Cupriavidus necator* can utilize CO_2, CO, or acetic acids based on the growth mode. Various experiments have demonstrated that substrate change does not affect the ability of *Cupriavidus necator* to produce polyhydroxyalkanoates, obtaining high cell dry weight of polyhydroxyalkanoates under autotrophic or heterotrophic modes in the region of 72–75% (Volova

et al. 2002; Garcia-Gonzalez and De Wever 2018). However, significant impact was seen on the polyhydroxyalkanoates composition: *Cupriavidus necator* was shown to produce a high polyhydroxybutyrate-co-valerate content with a 10–20 g/L feed of glucose (Możejko-Ciesielska and Kiewisz 2016), while it produced almost pure polyhydroxybutyrate under the presence of CO gas (Volova et al. 2002). Moreover, it can alternate between heterotrophic and autotrophic biosynthesis of polyhydroxyalkanoates or combine both modes.

Effect on Polyhydroxyalkanoates Production and Storage

For mixed cultures, limited studies have investigated systematically the influence of substrate composition on polyhydroxyalkanoates yield, in part because microbial mixed cultures vary as the substrate is altered, and hence studies typically focus on a particular substrate application. In a comparison of acetate and propionate as substrate, it was found the former led to higher polyhydroxyalkanoates storage (Lemos et al. 2006). In a separate study by Wang et al. investigating the combined role of dissolved oxygen and substrate with a microbial mixed culture, it was concluded that butyrate and valerate were consumed faster than acetic and propionic acids (Wang et al. 2017) under both high and low dissolved oxygen levels. It was also shown these two substrates were less affected by dissolved oxygen concentration, demonstrating interactions between various operational parameters and that low dissolved oxygen levels can be used with butyrate and valerate substrates, which will lower the costs of polyhydroxyalkanoates production. A few other studies have evaluated polyhydroxyalkanoates production by mixed cultures using other substrates such as butyrate, ethanol, glucose, malate, and others (Morgan-Sagastume et al. 2015; Duque et al. 2014; Cui et al. 2016). Duque et al. evaluated polyhydroxyalkanoates production by microbial mixed cultures using three types of wastes. Synthetic wastewater, which contained 68% acetate, 26% butyrate, 3% propionate, and 2% valerate; fermented sugarcane molasses, with a 38% propionate, 32% acetate, and 15% of valerate and butyrate content; and fermented cheese whey, with a content of 68% acetate, 21% butyrate, 8% propionate, and 3% lactate, were used to accumulate polyhydroxyalkanoates in microbial mixed cultures under the same conditions. The highest polyhydroxyalkanoates accumulation was achieved by fermented cheese whey reaching 65% cell dry weight, followed by fermented sugarcane waste that achieved a polyhydroxyalkanoates accumulation of 56% cell dry weight, while the synthetic wastewater resulted in an accumulation of 52% cell dry weight (Duque et al. 2014). Tamang et al. tested polyhydroxyalkanoates accumulation of a microbial mixed culture using acetate and acidified brewery wastewater under similar conditions. Acetate resulted in a higher polyhydroxybutyrate storage at 72.6% cell dry weight, while the wastewater resulted in 44.8% cell dry weight accumulation (Tamang et al. 2019). Complex substrates such as municipal wastewater and meat extract resulted in much lower accumulation of 15 and 13% cell dry weight, respectively, compared to values in the region of 40% cell dry weight achieved using acetate or propionate in the same study (Yuan et al. 2015). Therefore,

it may be necessary to add specific substrates to augment low-strength and complex wastewaters. Under mixed volatile fatty acids, feeding interactions between various volatile fatty acids is also key to consider, as well as the mode of operation and specific organisms that develop within the microbial mixed culture system.

In a pure culture experiment conducted by Mukhopadhyay et al., the effect of various acids on the accumulation of polyhydroxyalkanoates by *Rhodopseudomonas palustris* was evaluated under the same conditions. Acetate was found to produce the highest polyhydroxybutyrate content of 15.1% of cell dry weight, while malonate did not result in any polyhydroxyalkanoates accumulation. Other acids tested included malate, butyrate, citrate, and fumarate as well as glycerol, which accumulated 7.7%, 7.5%, 5.91%, 3.99%, and 3.41%, respectively (Mukhopadhyay et al. 2005). In a different experiment conducted by Yu et al., they evaluated the effects of various concentrations of butyrate and valerate on polyhydroxyalkanoates accumulation of *Cupriavidus metallidurans*. The results revealed that the highest polyhydroxyalkanoates accumulation of 0.41 g-polyhydroxyalkanoates/g-cell was achieved at a mixture of 80:20 butyrate/valerate, while the lowest accumulation of 0.06 g-polyhydroxyalkanoate/g-cell was achieved at 100% of valerate (Yu et al. 1998). The preference and ability to synthesize polyhydroxyalkanoates, however, varies from organism to organism. For instance, *Rhodopila globiformis*, an anoxygenic phototroph, does not convert acetate, but rather has a preference for ethanol and glucose (Imhoff et al. 2005). Hence, variations in substrate in a mixed culture will lead to changes in dominant organisms in the microbial community. New process conditions should therefore match the conditions suitable for enrichment of target organisms known to effectively store polyhydroxyalkanoates on a given substrate.

In certain instances, substrates can be inhibitory to polyhydroxyalkanoates production. For instance, Wang et al. demonstrated that butyrate and valerate can inhibit the conversion of acetate and propionate (Wang et al. 2018). In another study, Korkakaki et al. (Korkakaki et al. 2016) showed that in a mixed substrate influent of acetate and methanol only acetate led to polyhydroxyalkanoates accumulation, under the specific process environment, whereas methanol promoted non-polyhydroxyalkanoates storing organisms. By ending the feast phase and decanting the remaining methanol during the cycle, the authors managed to increase the accumulated cell polyhydroxyalkanoates from 48% to 70% wt. by preventing excess cell biomass growth on methanol (Table 5.2).

5.4.4 Feeding Regime

There are two groups of bacteria with the ability to produce polyhydroxyalkanoates. The first group creates polyhydroxyalkanoates in case of nutrient limitation such as oxygen, nitrogen, phosphate, or magnesium (Choi and Lee 1999). The second group does not demand nutrient limitation and can accumulate polyhydroxyalkanoates during the exponential growth phase (Chee et al. 2010). Generally, accumulation in

Table 5.2 Polyhydroxyalkanoates production via different strains and under various conditions

Feedstock	Strains	System	Polyhydroxyalkanoate content (%)	Monomer content Hydroxybutyrate:hydroxyvalerate	References
Acetate	Microbial mixed culture	Aerobic	30	100:0	Cui et al. (2016)
	Microbial mixed culture	Fed-batch	72.6	93:7	Tamang et al. (2019)
	Microbial mixed culture	Anoxic	35	N.a.	Dionisi et al. (2001)
	Microbial mixed culture	Aerobic	31	100:0	Beccari et al. (1998)
	Glycogen-accumulating organisms	Anaerobic/aerobic	41	93:7	Dai et al. (2007)
		Anaerobic	31	65:35	
Glucose	Microbial mixed culture	Aerobic	25	93:7	Kim et al. (1994) and Cui et al. (2016)
	Cupriavidus necator	Nitrogen-limiting	76	N.a.	Kim et al. (1994)
	Pseudomonas putida	Nitrogen-limiting	16.9	3-Hydroxydecanoate 3-Hydroxyhexanoate 3-Hydroxyoctanoate	Huijberts et al. (1992)
	Saccharophagus degradans	Fed-batch	52.8	N.a.	Sawant et al. (2017)
		Batch culture	25.3	N.a.	
Propionate and glucose	*Cupriavidus taiwanensis*	Flask	52	88:12	Sheu et al. (2009)
Glucose and fatty acids	*Escherichia coli*	Flask	28.7	3-Hydroxydecanoate 3-Hydroxydodecanoate	Hiroe et al. (2016)

Volatile fatty acid mixture – propionate and valerate	Microbial mixed culture	Aerobic	64	27.15% polyhydroxybutyrate 68.51% polyhydroxyvalerate 4.34% 3-hydroxy-2-methylvalerate	Hao et al. (2016)
Volatile fatty acid mixture – acetate, propionate, and lactate	Microbial mixed culture	Aerobic/ anaerobic	39	72:18	Dionisi et al. (2005)
Volatile fatty acid mixture – acetate and valerate	Microbial mixed culture	Aerobic	62	50:50	Beccari et al. (1998)
Volatile fatty acid mixture – acetate and propionate	Microbial mixed culture	Batch culture	35	N.a.	Villano et al. (2010)
Potato and valerate	*Cupriavidus taiwanensis*	Flask	55	90:10	Sheu et al. (2009)
Benzene	*Pseudomonas putida F1*	Flask	14	Various monomers: 3-Hydroxyoctanoate 3-Hydroxydecanoate 3-Hydroxydodecanoate 1,3-Hydroxydodecenoate	Nikodinovic et al. (2008)
Toluene	*Pseudomonas putida F1*	Flask	22		Nikodinovic et al. (2008)
Sucrose	*Azohydromonas lata*	No limitation	50	N.a.	Yamane et al. (1996)
Sucrose and soya	*Azohydromonas lata*	Batch fermentation	32.5	N.a.	Yu et al. (1999)
Whey	*Escherichia coli* with *Azohydromonas lata*	Fed-batch	80.2	N.a.	Ahn et al. (2000)
Food waste	Microbial mixed culture	Anaerobic/ aerobic	51	96:4	Rhu et al. (2003)

(continued)

Table 5.2 (continued)

Feedstock	Strains	System	Polyhydroxyalkanoate content (%)	Monomer content Hydroxybutyrate:hydroxyvalerate	References
Beet molasses	*Azotobacter vinelandii*		60	N.a.	Page (1989)
Fermented molasses	Microbial mixed culture	Pulse feeding	56	85:15	Albuquerque et al. (2011)
Paper mill effluents	Glycogen-accumulating organisms – *Defluviicoccus vanus* – *Candidatus Competibacter phosphatis*	Anaerobic/ aerobic	42	6:47:47 (hydroxymethylvalerate)	Bengtsson et al. (2008)
		Aerobic	22	51:35:14 (hydroxymethylvalerate)	
Wastewater	Microbial mixed culture	Anaerobic/ aerobic	53	50:30	Coats et al. (2007)
Calophyllum inophyllum oil cake	*Enterobacter aerogenes* *Rhodobacter sphaeroides*	Photo/dark fermentation	60.3	100:0	Arumugam et al. (2014)
Waste frying oil	*Cupriavidus necator*	No limitation	62.5	N.a.	Kahar et al. (2004)
	Burkholderia thailandensis	Batch fermentation	60	100:0	Kourmentza et al. (2018)
Textile dye direct red 5B	*Sphingobacterium*	Fermentation	64	3-Hydroxyhexadecanoate	Tamboli et al. (2010)
Cornstarch and soybean oil waste	*Escherichia coli*	No limitation	5.9	0.5% polyhydroxybutyrate 44.9% PHHx 54.6% 3-hydroxyoctanoate	Fonseca and Antonio (2006)
Palm kernel oil	*Cupriavidus necator*	Batch fermentation	87	95% polyhydroxybutyrate 5% PHHx	Loo et al. (2005)
Sugarcane bagasse waste	*Cupriavidus necator*	Nitrogen-limiting	53.7%	70:30	Yu and Stahl (2008)

Brewery wastewater	Microbial mixed culture	Acidified	44.8	59:41	Tamang et al. (2019)
	Microbial mixed culture	Anaerobic	43.7	68:32	
Cassava starch wastewater	*Bacillus tequilensis*	Sequencing batch reactor	79.2	N.a.	Chaleomrum et al. (2014)
Methanol	*Methylobacterium organophilum*	Fed-batch	52.0	N.a.	Kim et al. (1996)
Methane	*Methylotroph* spp.	Batch culture	51.0	N.a.	Wendlandt et al. (2001)

the latter group of organisms is triggered through a feast-famine regime in a fed-batch fermenter. The first group includes bacteria such as *Cupriavidus necator* and *Pseudomonas,* while the second group includes *Azohydromonas lata* and *Escherichia coli* (Muralidharan et al. 2013).

Feeding regime affects the polyhydroxyalkanoates yield as well as the monomer composition. Under transient feeding, where specific amount of carbon substrate is supplied periodically, the organism experiences periods of feast, where there is external substrate availability; and famine, where there is no available external substrate. When the biomass is subjected to successive periods of feast and famine, it experiences an unbalanced growth. This approach is considered the most promising due to the high polyhydroxyalkanoates accumulation as it promotes the conversion of carbon substrate to polyhydroxyalkanoates over other intracellular materials (Salehizadeh and van Loosdrecht 2004). Additionally, the bacteria that contain polyhydroxyalkanoates showed improved tolerance against transient environmental changes (Tan et al. 2014). The biomass is forced to adapt in order to survive and grow in a famine environment. This naturally selects for polyhydroxyalkanoate-accumulating organisms that can convert substrate to polyhydroxyalkanoates during the feast period and maintain an overall higher average growth rate over the feast and famine periods through growth on stored polyhydroxyalkanoates (Valentino et al. 2016). Typically, the feast-famine cycle is started out with long famine duration to create a strong selection pressure for polyhydroxyalkanoates accumulating organisms and is then decreased to increase the overall rate of substrate addition and polyhydroxyalkanoates production prior to harvest.

The feast-famine regime usually produces suitable conditions for the storage of volatile fatty acids as polyhydroxyalkanoates since they are rapidly converted by the bacteria (Basset et al. 2016), with polyhydroxyalkanoates accumulation using microbial mixed cultures and volatile fatty acids as substrate producing polyhydroxyalkanoates contents of up to 90% cell dry weight (Korkakaki et al. 2016; Johnson et al. 2009a).

In an experiment comparing the effect of feeding mode, the semicontinuous pulse feeding produced a 64.5% polyhydroxyalkanoates content from an activated sludge from wastewater treatment and food waste plant with fermented volatile fatty acids as substrate, while the one-time pulse-feeding mode was able to produce only 51.5% polyhydroxyalkanoates content (Chen et al. 2013). Moreover, the semicontinuous feeding was able to produce more diverse monomers compared to the feast-famine mode (Albuquerque et al. 2011). These experiments illustrated that a more frequent pulse-feeding mode with a short cycle will result in more diverse polyhydroxyalkanoates and a higher yield. In an experiment conducted by Marang et al., it was revealed that the feeding regime impacts significantly the accumulation of polyhydroxyalkanoates. They tested a continuous feed, which resulted in the accumulation of 53% cell dry weight of polyhydroxyalkanoates, while a true feast feed where 50% of the substrate was supplied at the beginning led to a higher accumulation of 85% cell dry weight (Marang et al. 2018). Moreover, organic loading rate and sludge retention also play an important role when comparing the impact of feeding mode. The improvement of feast-famine over continuous feeding is much

more notable at longer sludge retention time and lower organic loading rates (Ciggin et al. 2012). In the study of Ciggin et al., notable differences in polyhydroxyalkanoates production were observed at organic loading rates of roughly 1000 mg-COD/L/d and a sludge retention time of 8 days. These differences were reduced when the sludge age was reduced to 2 days and were negligible when the organic loading was increased to roughly 3000 mg-COD/L/d.

Organic loading rate has also been demonstrated to impact the polyhydroxyalkanoates monomer composition. Chen et al. showed that, under continuous feeding, the rate of biomass loading had a significant impact on the hydroxybutyrate:hydroxyvalerate ratio, such that at around 24 C-mol volatile fatty acids/C-mol X/d, the hydroxybutyrate:hydroxyvalerate ratio was minimal and was around half that observed at the lowest loading rate of 4 mol volatile fatty acids/C-mol X/d. The hydroxybutyrate:hydroxyvalerate ratio increased slightly from the minimum ratio at higher biomass feeding rates (Chen et al. 2015).

A significant issue with volatile fatty acid–rich wastewater under pulse feed (feast) type operation is the high transient concentrations of volatile fatty acids present in the reactor. Under such cases if the pH is low, unionized volatile fatty acids may inhibit the microbial culture. Chen et al. (Chen et al. 2015) found that when operating a system at relatively high volatile fatty acid concentration (115–120 mM), continuous feeding provided improved accumulation and yields compared with batch feed at all pH tested, but particularly at the acidic pH of 5 where continuous feed provided the highest value while for batch feed accumulation and yields were lowest. Moreover, at lower biomass loading rates, polyhydroxyalkanoates accumulation was found to be less sensitive to all influent pH values tested, all likely as a result of reduced inhibition.

5.4.5 Nutrient Limitation

Nutrient limitation is when sources of nitrogen or phosphate are depleted in the environment. Under nitrogen-limiting conditions, protein synthesis is halted, which in turn stops the progress of the Krebs cycle enzymes such as citrate synthase, responsible for incorporating acetyl-CoA into the energy generating metabolic cycle. As a result, the excess acetyl-CoA, a metabolic precursor to polyhydroxybutyrate, is instead directed toward this intracellular storage product (Muralidharan et al. 2013). It has been observed under nitrogen-limiting conditions, that the bacterial cells do not increase in number, but rather increase in weight and size (Chee et al. 2010), which is explained by the fact that the polyhydroxyalkanoates production increases during this stage. Additionally, when nutrient-limiting conditions are coupled with the feast-famine strategy, they enable the polyhydroxyalkanoates-producing bacteria to maximize nutrient resources as they adjust to the new environmental conditions (Tan et al. 2014).

The carbon to nutrient ratio is an important variable that affects the production of polyhydroxyalkanoates. Studies concluded that polyhydroxyalkanoates production

was higher under nitrogen-limiting conditions rather than excess of nitrogen or no nitrogen availability (Saharan et al. 2014; Montiel-Jarillo et al. 2017). Optimal values vary depending on the culture, but are typically in the range of 12 to 25 g-C/g-N (Silva et al. 2016). Silva et al. found that the timing of nutrient addition to the system played an important role in both the accumulation and the composition of stored polyhydroxyalkanoates. When nitrogen was added during the famine phase (uncoupled strategy) rather than during the feast phase (coupled strategy), polyhydroxyalkanoates production increased more than twofold reaching 1300 mg-COD/L, while the hydroxyvalerate monomer content increased by 82% reaching a content of up to 20% wt/wt. Content of hydroxyvalerate monomer showed a decrease, however, with increasing C/N ratio, while stored polyhydroxyalkanoates also showed a significant deterioration when the C/N ratio increased from 14.3 mol-C/mol-N to 22.3 mol-C/mol-N. Yield and cell storage as cell dry weight both roughly doubled under the uncoupled nutrient addition strategy. Under this strategy ammonia was uptaken during polyhydroxyalkanoates degradation for cell biomass growth, promoting a stronger selection pressure for polyhydroxyalkanoates accumulation (Silva et al. 2016). In another experiment conducted by Chua et al. treating a xenobiotic wastewater in an activated sludge system, different types of polyhydroxyalkanoates monomers were produced from wastewater under nitrogen-limiting conditions. With an increasing C/N ratio from 20 to 140, the cell storage of polymer increased from 11% to 39% of cell dry weight (Chua and Yu 1999). Chua and Yu also reported changes in the copolymer ratios based on the C/N ratio in the microbial mixed culture.

Phosphorus limitation can also be used to control the behavior of polyhydroxyalkanoates production. In chemostat-based systems, phosphorus limitation acts in a similar manner to nitrogen limitation, preventing cell biomass growth and forcing carbon toward intracellular storage products (Cavaillé et al. 2016). Careful control of phosphorus concentration and addition can therefore direct carbon toward cell growth or polyhydroxyalkanoates. Moreover, the authors found that at lower dilution rates (i.e., lower critical growth rates), the cultures were more adaptive to higher phosphorus limitation. In contrast, in a sequencing batch reactor, it was found that phosphorus limitation led to reduced substrate conversion (Korkakaki et al. 2017). At a C/P ratio of 150 C-mol/P-mol, no significant impact on accumulation was noticed, but at 300 C-mol/P-mol, polyhydroxyalkanoates accumulation was severely affected. The relation of substrate conversion vs P limitation allows control over oxygen uptake rate in the system and therefore peak air demands required by the aeration system.

5.4.6 pH Impact

According to many studies, pH plays an important role in the production of polyhydroxyalkanoates inside the cells (Montiel-Jarillo et al. 2017), affecting the accumulation of polyhydroxyalkanoates as well as the monomer composition when the pH

is controlled. Hashimoto et al. explored the impact of pH on polyhydroxyalkanoates production in *Rhodospirillum rubrum* grown in an acetate environment. The highest polyhydroxyalkanoates accumulation reached 0.67 g-polyhydroxyalkanoates/g-dry cell at pH 7 (Hashimoto et al. 1993). For pure cultures, experiments were conducted on *Pseudomonas aeruginosa.* The highest yield of polyhydroxybutyrate of 60% cell dry weight was reached at a pH of 7 with peptone as a nitrogen source (Muralidharan et al. 2013). A research conducted by Gomaa et al. evaluated the impact of pH on polyhydroxyalkanoates production on *Bacillus subtilis* and *Escherichia coli* in similar environments. The highest polyhydroxyalkanoates accumulation was observed at pH of 7 at 62.2% and 58.7% for *Bacillus subtilis* and *Escherichia coli*, respectively (Gomaa 2014).

An experiment conducted by Amulya et al. on the impact of pH for the production of polyhydroxyalkanoates in various microbial mixed culture environments revealed that a pH of 7 was more beneficial to the dehydrogenase enzyme activity and substrate degradation compared to acidic and alkaline conditions in a microaerobic environment. These conditions showed an increase in polyhydroxyalkanoates accumulation to 56% at pH 7 compared to lower polyhydroxyalkanoates accumulation in acidic and basic environments (Amulya et al. 2016). Experiments of pH impact on polyhydroxyalkanoates production with microbial mixed culture under nitrogen-limiting conditions revealed that environments with no-pH control led to very alkaline conditions and produced up to 44% of polyhydroxyalkanoates cell dry weight. With pH control between 8.8 and 9.2, the polyhydroxyalkanoates content increased up to 51% polyhydroxyalkanoates cell dry weight with a pH value ranging from 8.8 to 9.2 (Montiel-Jarillo et al. 2017). Moreover, both yield and polyhydroxyalkanoates production rates also increased with increasing pH. Other studies conducted on microbial mixed cultures producing polyhydroxyalkanoates revealed a production of higher amounts of hydroxyvalerate monomers in case of pH 9.5 compared to a pH of 5.5 under the same conditions (Dionisi et al. 2005). Pittman et al. conducted an experiment on wastewater as a substrate and evaluated the effects of pH on the production. A pH of 8 was the best operating condition for a high and stable production of polyhydroxyalkanoates achieving 28.4% of cell dry weight, whereas a pH of 6 halted the accumulation of polyhydroxyalkanoates inside the cells (Pittmann and Steinmetz 2014).

5.4.7 Temperature Effect

Very few studies evaluated the impact of temperature on polyhydroxyalkanoates production. However, based on various experiments, the ideal temperature of polyhydroxyalkanoates production varies depending on the type of bacterial strains involved in the process and seems to range from ambient temperature to 30 °C. Experiments conducted on the effects of temperature on microbial mixed culture growth and polyhydroxyalkanoates accumulation have shown that the ideal polyhydroxyalkanoates accumulation occurs at a temperature of 15 °C when fed

with pure acetate (De Grazia et al. 2017). A similar experiment conducted on microbial mixed cultures observed that polyhydroxyalkanoates accumulation rate reduced as the temperature increased (Krishna and Van Loosdrecht 1999). Similar trends were later reported by Jiang et al. using a microbial mixed culture as well (Jiang et al. 2011c). Johnson evaluated the impact of temperature in a short-term and long-term fermenter. The temperature was varied from 15 to 35 °C in a sequencing batch reactor with a feast-famine mode to monitor short-term effects. The study concluded that there was a little variation in polyhydroxyalkanoates production in the fermenter with short-term feeding and temperatures ranging from 20 to 35 °C. However, a low temperature of 15 °C resulted in the highest polyhydroxyalkanoates production. However, for the long-term feeding operated fermenter, the highest polyhydroxyalkanoates production was achieved at 30 °C, as a result of a change in the microbial community (Johnson et al. 2009b). The new community at higher temperatures had much more rapid conversion of substrate and longer famine periods than at the lower temperature steady states. Jiang et al. investigated the effects of temperature on polyhydroxybutyrate production under feast-famine conditions. They conducted the experiment on *Zoogloea* and *Plasticicumulans acidivorans* bacteria in the range of 20 to 30 °C in separate batches. Both bacteria produced more than 75% polyhydroxyalkanoates cell dry weight at the optimal condition of 20 and 30 °C for *Zoogloea* and *Plasticicumulans acidivorans,* respectively (Jiang et al. 2011c). Temperature is therefore highly dependent on culture and the selected organism(s) responsible for polyhydroxyalkanoates production.

5.4.8 Light Impact

Sunlight functions as an energy source to drive carbon conversion, whether autotrophically from CO_2 or heterotrophically from organic carbon feedstocks for oxygenic and anoxygenic phototrophs, respectively. Photosynthetic bacteria are explored as a means to reduce costs associated with aeration (Fradinho et al. 2013b) by utilizing abundant availability of sunlight to supply energy for carbon conversion. Light-dark cycling is reported to regulate the accumulation of polyhydroxyalkanoates in some types of bacteria under anoxygenic or anaerobic conditions, whereby carbon is stored during photosynthesis and then consumed for energy during dark hours (Saharan et al. 2014; Fradinho et al. 2013b).

Cyanobacteria, such as *Arthrospira* (formerly known as *Spirulina*), are known photosynthetic bacteria with the ability to produce polyhydroxyalkanoates (Chee et al. 2010). *Synechococcus* was able to produce up to 55% polyhydroxyalkanoates of cell dry weight under phosphate-limiting conditions, whereas, *Synechocystis* was able to accumulate 15% of polyhydroxyalkanoates of cell dry weight under nitrogen-limiting conditions (Saharan et al. 2014). It was discovered that one of the best triggers for polyhydroxyalkanoates accumulation in cyanobacteria is nutrient-limiting conditions because nitrogen-depleted cells do not have the ability to synthesize proteins needed for reproduction, which triggers the accumulation of

polyhydroxyalkanoates (Troschl et al. 2017). One of the limitations of cyanobacteria is their production of only scl-polyhydroxyalkanoates (Chee et al. 2010; Troschl et al. 2017) (Table 5.3).

In an experiment where the culture was grown under high light intensity and then moved to a lower light intensity environment, the authors noticed a switch in the activities of microbial mixed cultures from polyhydroxyalkanoates production to growth. Based on these results, the same group evaluated the impact of light intensity further. It was revealed that there is a direct correlation between light intensity and polyhydroxyalkanoates production rate where the latter increased as the light intensity increased. Acetate uptake rate, on the other hand, increased initially until it reached light intensity of 18.5 W/g X and decreased thereafter. The selected light intensity of 18.5 W/g X is close to that provided by sunlight, which should be taken into consideration for further scale-up (Fradinho et al. 2019).

Fradinho et al. were able to accumulate polyhydroxyalkanoates using a mixture of photosynthetic bacteria and microalgae under cyclic feeding and constant anaerobic illuminated conditions up to a content of 20% (Fradinho et al. 2013a). In a separate experiment conducted by the same research group, they produced polyhydroxyalkanoates under light cycling and feeding cycling conditions in an anaerobic atmosphere using a photosynthetic microbial mixed culture (Fradinho et al. 2013b). Feeding took place simultaneously with the dark phase, while the light phase was accompanied with a famine period. This experiment selected for anoxygenic phototrophs rather than cyanobacteria and was able to accumulate polyhydroxyalkanoates up to 30% of polyhydroxyalkanoates content with acetate as a carbon source (Fradinho et al. 2013b). Improved selection of anoxygenic phototrophs and improved accumulation and productivity were achieved under a continuous feeding and lighting regime. Notably, it was found that higher light intensity improved polyhydroxyalkanoates accumulation as the organisms require light to drive the polyhydroxyalkanoates synthesis process.

Table 5.3 Polyhydroxyalkanoates-producing cyanobacteria and polyhydroxyalkanoates content

Strain of cyanobacteria	Polyhydroxyalkanoates content (%)	References
Arthrospira subsalsa (formely *Spirulina*)	14.7	Shrivastav et al. (2010)
Arthrospira platensis	22	De Morais et al. (2015)
Synechococcus	55	Nishioka et al. (2001)
Synechocystis PCC6803	11	Panda and Mallick (2007)
Synechocystis PCC6803	26	Khetkorn et al. (2016)
Nostoc muscorum Agardh	27	Bhati and Mallick (2016)
Nostoc muscorum	35	Sharma and Mallick (2005)
Nostoc muscorum Agardh	78	Bhati and Mallick (2015)

5.5 Microbial Community Dynamics

Interactions between microbial communities are of high importance in microbial mixed culture systems. In a system studying a change in feedstock, it was found both the microbial communities of the fermentation step and polyhydroxyalkanoates accumulation steps in a three-stage system underwent significant changes when the feedstock changed from molasses to cheese whey, with higher hydroxyvalerate precursors during feeding with molasses. Despite this, both acidogenic and polyhydroxyalkanoates production performance were similar after pseudo-steady-state acclimation demonstrating functional redundancy in the system (Carvalho et al. 2018).

Experiments conducted by Albuquerque et al. revealed that bacterial strains have preferences to the substrates they consume. For instance, *Azoarcus* consumed acetate, *Thauera* consumed butyrate, while *Paracoccus* consumed a larger range of substrates (Albuquerque et al. 2012). In various experiments, it was revealed that *Thauera* is linked to the production of hydroxyvalerate monomers (Carvalho et al. 2014), while *Paracoccus* is able to accumulate polyhydroxybutyrate, polyhydroxyvalerate, and polyhydroxybutyrate-co-valerate when fed with methanol, pentanol, and a mixture of methanol and pentanol, respectively (Yamane et al. 1996; Maehara et al. 2001). Wang et al. studied the effect of different organic loading rates, sludge retention time, and dissolved oxygen on the startup of polyhydroxyalkanoates producing sequencing batch reactor systems operated under feast-famine regime (Wang et al. 2017). One reactor was operated with a high organic loading rate, high dissolved oxygen (3.47 mg/L), and sludge retention time of 4 days, while the second was initially operated under a low dissolved oxygen (0.87 mg/L) and organic loading rate with an 8 d sludge retention time. The organic loading rate was gradually increased to that of the first reactor and then the sludge retention time reduced to 4 days also. It was found that the initial and final microbial communities were very similar in composition despite the differing culture conditions with time and dissolved oxygen concentrations and were dominant in *Plasticicumulans*, *Zoogloea*, *Paracoccus*, and *Flavobacterium*, suggesting dissolved oxygen did not largely impact the microbial community. However, at the midpoint of the experiment, where organic loading rate was similar but the sludge retention time was still varied, the microbial community in the second sequencing batch reactor was dominated by *Amaricoccus* and *Azoarcus*, polyhydroxyalkanoates producers that are known to require a longer sludge retention time (Wang et al. 2017).

5.6 Process Design

A variety of processes exist that can produce polyhydroxyalkanoates by mixed and pure cultures. The selection of the process will be dependent on the nature of the feedstock (or wastewater), expected microbial community competition, and

location-dependent economic and environmental constraints, such as the cost of energy. In general, a microbial mixed culture requires three steps: (1) fermentation of complex substrate to readily biodegradable substrates such as volatile fatty acids, (2) culture enrichment, and (3) polyhydroxyalkanoates accumulation. In certain circumstances, enrichment and accumulation phases may be possible to perform together. A summary of the various overarching modes of process design is described in Table 5.4 for enrichment and accumulation.

The anaerobic-aerobic method was the first method discovered to induce polyhydroxyalkanoates storage and was associated with biological phosphorus removal. Depending on the nature of the substrate and whether fermentation is required or sufficient in the anaerobic stage, a preceding fermenter may be necessary. The anaerobic-aerobic method has the advantage that aeration is reduced, especially if the famine phase is conducted under anoxic conditions. While accumulation is typically low, values up to 60% cell dry weight have been reported (Albuquerque et al. 2007) and have the added benefit that it can be coupled with phosphorus removal. For compatibility with existing treatment processes, the anaerobic-aerobic method may be a preferred choice. Aerobic-anoxic and anoxic-aerobic systems rely on similar integration with existing wastewater treatment processes where in the former denitrification occurs on stored polyhydroxyalkanoates. Both can reduce aeration requirements. While the former has achieved limited polyhydroxyalkanoates storage (Kourmentza et al. 2017), anoxic-aerobic systems have reported accumulation as high as 60% g-polyhydroxyalkanoates/g-VSS (Anterrieu et al. 2014). Such systems have been enhanced through integration with fixed-film processes such as moving bed biofilm reactor carriers or integrated fixed-film activated sludge systems to separate the long sludge retention times required for nitrification where this process is still required for treatment from the short sludge retention times desired for high-rate polyhydroxyalkanoates production (Anterrieu et al. 2014; Bengtsson et al. 2017).

The three-step process is another type of continuous flow process. The first step consists of conversion of carbohydrates to volatile fatty acids under anaerobic fermentation. This step is often carried out under acidic conditions to help promote acidogenic bacteria and ensure inhibition of methanogenic bacteria that will compete for acetate that has been produced. The second step of the process consists of the enrichment of activated sludge for the production of polyhydroxyalkanoates-accumulating bacteria, most often, under aerobic dynamic feeding (ADF) conditions. The last step of the process consists of the polyhydroxyalkanoates accumulation process under aerobic conditions in a fed-batch reactor (Kourmentza and Kornaros 2016). Aerobic dynamic feeding methods are the most commonly reported methods from studies to induce polyhydroxyalkanoates accumulation and in general achieve higher accumulation levels than the methods relying on alternating redox conditions with values of 90% cell dry weight being reported (Jiang et al. 2011d). The alternating feast-famine conditions promote cultures with strong ability to produce polyhydroxyalkanoates during substrate-rich conditions.

One issue with aerobic dynamic feeding systems is the high aeration requirements and the need for sufficient famine conditions that may reduce overall

Table 5.4 Summary of various process design approaches to promote polyhydroxyalkanoates accumulation

Process	Phase	Aeration	Electron/acceptor	Energy supply	Carbon supply	Nutrient availability	Cause for polyhydroxyalkanoates accumulation
Anaerobic-aerobic	Feast Famine	No Yes	Polyhydroxyalkanoate O_2/NO_3^-	Glycogen/ polyphosphate Polyhydroxyalkanoates oxidation	External substrate Polyhydroxyalkanoates	Yes Yes	Lack of electron/acceptor Transient substrate
Aerobic dynamic feeding (ADF)	Feast Famine	Yes Yes	O_2 O_2	Substrate oxidation Polyhydroxyalkanoates oxidation	External substrate Polyhydroxyalkanoates	Yes Yes/no	Transient substrate
ADF with intermediate settling	Feast Famine	Yes Yes	O_2 O_2	Substrate oxidation Polyhydroxyalkanoates oxidation	External substrate Polyhydroxyalkanoates	Yes Yes	Transient substrate Settling pressure Elimination of COD from famine phase
ADF with feast-phase N limitation	Feast Famine	Yes Yes	O_2 O_2	Substrate oxidation Polyhydroxyalkanoates oxidation	External substrate Polyhydroxyalkanoates	No Yes	Transient substrate N limitation during feast phase
Photosynthetic sequencing batch reactor	Feast Famine	No No	Polyhydroxyalkanoate Algal O_2	Light Polyhydroxyalkanoates oxidation + light	External substrate Polyhydroxyalkanoates	Yes Yes	Lack of external electron/ acceptor in light Transient substrate
Photosynthetic dark feast phase	Feast Famine	No No	Polyhydroxyalkanoate Algal O_2	Glycogen Polyhydroxyalkanoates oxidation + light	External substrate Polyhydroxyalkanoates	Yes Yes	Lack of external electron/ acceptor in light Transient substrate
Photosynthetic permanent feast	Feast only	No	Polyhydroxyalkanoate	Light	External substrate	Yes	Lack of external electron/ acceptor in light
Aerobic-anoxic	Feast Famine	Yes No	O_2 NO_3/NO_2	Substrate oxidation Polyhydroxyalkanoates oxidation	External substrate Polyhydroxyalkanoates	Yes Yes	Transient substrate

Anoxic-aerobic	Feast Famine	No Yes	NO_3/NO_2 O_2	Substrate oxidation Polyhydroxyalkanoates oxidation	External substrate Polyhydroxyalkanoates	Yes Yes	Transient substrate
Microaerophilic	Feast Famine	Yes Yes	Limited O_2 Limited O_2	Substrate oxidation Polyhydroxyalkanoates oxidation	External substrate Polyhydroxyalkanoates	Yes Yes	Transient substrate Limited electron/acceptor

Adapted from Kourmentza et al. (2017)

volumetric productivity and conversion yield. The aerobic dynamic feeding method has been further enhanced through incorporation of nutrient limitation during the feast phase to promote polyhydroxyalkanoates synthesis over growth (Silva et al. 2016). In a separate modification, the incorporation of an intermediate settling phase provides a selective pressure based on the higher density of polyhydroxyalkanoates-rich biomass to washout non-polyhydroxyalkanoates storing biomass and allows the decanting of remaining soluble organics at the end of the feast phase to prevent non-polyhydroxyalkanoates growth in the famine phase (Chen et al. 2016). Zeng et al. demonstrated the value of settling selection in the enrichment phase, reaching steady-state polyhydroxybutyrate content in two thirds the time of standard aerobic dynamic feeding. Furthermore, they showed that a feed-on-demand approach using oxygen uptake rate measurements further improved storage from 70% to 83% cell dry weight, yield from 0.6 to 0.81 Cmol-polyhydroxybutyrate/CmolAc, and specific polyhydroxybutyrate production from 0.43 to 0.85 Cmol-polyhydroxybutyrate/Cmol-X/h (Zeng et al. 2018).

For microbial mixed culture aerobic dynamic feeding processes, the sequencing batch reactor is a preferred reactor system. Sequencing batch reactors are easy to control, highly flexible and allow quick modifications of the process conditions. Moreover, sequencing batch reactors allow control of process parameters with time, including substrate concentration and duration profiles and, therefore, are suitable for time-based feast-famine process control, allowing control over structure and functions of the composed microbial community (Salehizadeh and van Loosdrecht 2004). They can be operated under intermittent substrate feeding in order to favor the storage of the polymer by the sludge. For instance, fed-batch fermentation was proven to be more efficient in producing high cell concentration, due to the ability of controlling substrate inhibition in the medium (Chee et al. 2010).

In some instances, particularly with highly inhibitive substrates, stepwise fed-batch modes are more likely to prevent inhibition and increase polyhydroxyalkanoates accumulation (Kourmentza et al. 2017; Albuquerque et al. 2007). Continuous flow–based systems may be even more advantageous with inhibitive substrates. Such systems also reduce the reliance on integrating multiple reactors to manage continuous flows typical of most wastewater systems. One two-step continuous flow layout is to combine a plug flow reactor (PFR) with a subsequent continuous flow stir tank reactor (CSTR) and settler returning concentrated biomass to the plug flow reactor. The plug flow reactor promotes feast conditions, while the continuous flow stir tank reactor provides famine-like conditions (Chee et al. 2010; Salehizadeh and van Loosdrecht 2004). Contact-stabilization configuration with biomass recycle is another continuous flow setup that promotes continuous polyhydroxyalkanoates production. Sarioglu et al. used a contact-stabilization configuration with two aerated reactors, contact and stabilization reactors, separated with a sedimentation tank. The influent wastewater enters the contact reactor and moves to the sedimentation basin. The overflow of the sedimentation basin leaves as treated effluent, while the underflow moves to the stabilization reactor, which goes back to the contact aerator (Sarioglu et al. 2003). Similar to the plug flow reactor coupled with continuous flow stir tank reactor, the recirculation of the biomass results in a continuous

accumulation of polyhydroxyalkanoates. Continuous flow (feed) systems have the benefit that polyhydroxyalkanoates accumulation occurs continuously and can therefore lead to higher volumetric production rates of polyhydroxyalkanoates (Kourmentza et al. 2017).

Regardless of the type of system selected, it is important to note that the economics and feasibility of the process are driven by three key factors, namely, a high carbon to polymer yield (particularly if the substrate is costly), a high volumetric productivity resulting in small reactors and high cell densities, and high intracellular storage content leading to reduced residual waste. An example of a microbial mixed culture system highlighting this point is that of Chen et al. (Chen et al. 2015). In their study they used a continuous feeding mode and found at low biomass loading rates polyhydroxyalkanoates production was enhanced to a maximum of 70.4% of cell dry weight. However, lower loading rates lead to less biomass production, reducing overall productivity, and dilute suspension cultures, making subsequent processing of the polymer costly. Chua et al.'s study on using nutrient limitation also highlights this consideration (Chua and Yu 1999). While high C/N ratios led to the maximum cell dry weight of 39% at a C/N ratio of 140, increasing nutrient limitation decreased cell growth such that the maximum overall polyhydroxyalkanoates productivity was achieved at a C/N ratio of 100. From a systems perspective, the optimum ratio would then lie between 100 and 140, depending on cell harvesting and polymer extraction costs and their relationship to bioreactor cell concentrations. Process design is further complicated by the economic value of the polymer based on its monomer composition, which may change as the process design changes. Given the limited number of pilot microbial mixed culture systems, this is an area where significant scope exists for further research and may be aided through computer-aided process design.

5.7 Polyhydroxyalkanoates Quantification

Determining the presence of polyhydroxyalkanoates and its quantity in polyhydroxyalkanoates-producing microbial cells can be done through various methods and is important for monitoring the performance of the polyhydroxyalkanoates production process. The most widely used method is gas chromatography (GC) with either mass spectrometry (MS) detection or flame ionization detection (FID) as they are highly sensitive and accurate detection methods. Both methods require depolymerization and conversion into derivatives such as acids or methyl ester, but these methods provide detailed analysis of polyhydroxyalkanoates monomers, which is useful in process optimization (Tan et al. 2014; Koller et al. 2011). On average, one test of GC-MS is completed in 4 hours (Godbole 2016). Nuclear magnetic resonance (NMR) spectroscopy is another method that enables the understanding of polyhydroxyalkanoates monomeric composition (Tan et al. 2014). It does not require the hydrolysis of the polymer (Godbole 2016). Additionally, it can

be combined with polarization magic-angle spinning to avoid the cell lysis step (Jacob et al. 1986).

Colony and/or cell staining is a popular method where Nile red, Nile blue A, or Sudan black dye is added into the growth medium. The staining method followed by fluorescent microscopy is used for visualization of polyhydroxyalkanoates when Nile red or Nile blue A is used, or visible light microscopy with Sudan black. The dyes are more soluble in polyhydroxyalkanoates and, therefore, make the cells more visible (Koller et al. 2011). While this method is rapid and suitable for a large number of samples, it is prone to inaccuracies as other lipophilic non-polyhydroxyalkanoate-containing groups could be stained in the process (Koller et al. 2017). Transmission electron microscopy (TEM) allows direct visualization of polyhydroxyalkanoates, but requires the use of radioactive chemicals for sample preparation and is time-consuming (Tan et al. 2014). These mentioned methods allow the detection of polyhydroxyalkanoates-producing microorganisms, but cannot quantify the amount of polyhydroxyalkanoates produced nor its monomeric composition on their own. Many experiments have coupled these methods with image analysis in order to extract the amount of polyhydroxyalkanoates accumulation in cells (Elain et al. 2015; Takahashi et al. 2017).

Gravimetric methods are among the first employed methods to evaluate the quantity of polyhydroxyalkanoates produced. First, chloroform is used to extract polyhydroxyalkanoates from lyophilized bacterial cells, and the acetone or diethyl ether is used for the precipitation of polyhydroxyalkanoates. Turbidity measurements can also predict polyhydroxyalkanoates amounts after lysis of cell material (Koller et al. 2011). Flow cytometry, spectrofluorometry, and Fourier transform infrared (FTIR) spectroscopy are all methods that allow to measure polyhydroxyalkanoates quantity without breaking cell walls (Koller et al. 2011). Moreover, FTIR has the advantage of detecting different types of polyhydroxyalkanoates monomers without use of hazardous chemicals in a short time and no cell lysis is required either (Tan et al. 2014). Flow cytometry and spectrofluorometry provide results after 5 to 30 min (Godbole 2016; Alves et al. 2017). Of these methods, staining techniques using fluorescence-based quantification such as flow cytometry or spectrofluorometry are most suitable for online analysis at a treatment plant.

5.8 Polyhydroxyalkanoates Processing and Recovery

Polyhydroxyalkanoates are an intracellular product stored inside the cells. Therefore, its separation and harvesting methods are expensive and complex. There are five steps involved in recovery of polyhydroxyalkanoates, which start by harvesting, followed by an optional pretreatment, then retrieval, accumulation, and finally purifying and drying (Kourmentza et al. 2017; Tan et al. 2014). Figure 5.6 summarizes the steps followed to recover polyhydroxyalkanoates granules.

The first step is harvesting, and it consists of collecting the biomass from the fermenter via centrifugation or filtration (Kourmentza et al. 2017). This step aims at

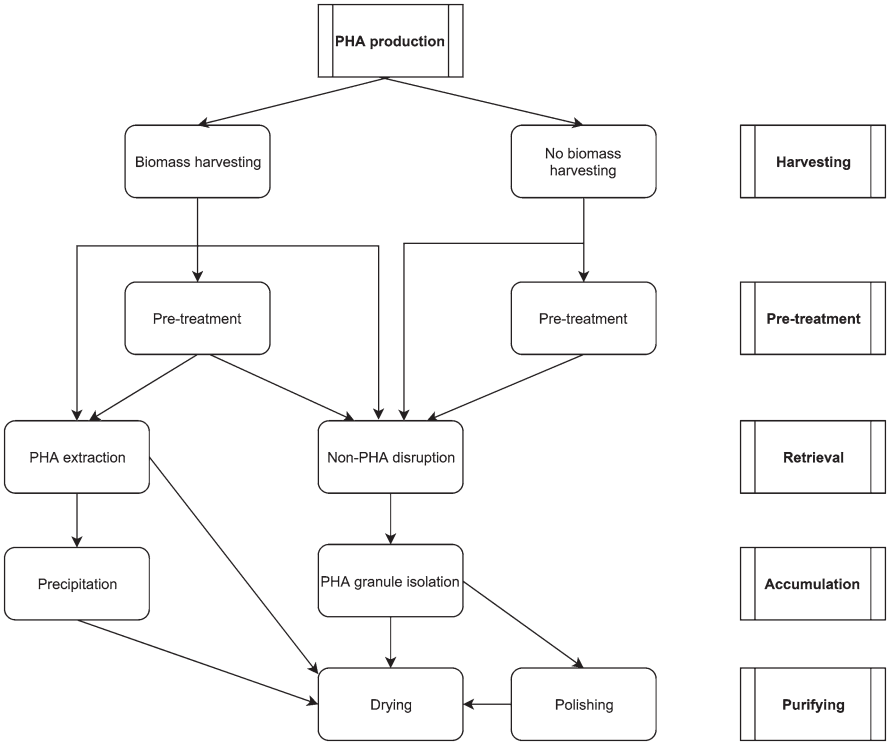

Fig. 5.6 Polyhydroxyalkanoates recovery system

concentrating the biomass to facilitate the recovery process. The pretreatment step aims at simplifying the retrieval of polyhydroxyalkanoates in the next steps by weakening the cell walls. Pretreatment methods are various and include lyophilization; mechanical, chemical, and biochemical treatments; thermal drying; and grinding (Kourmentza et al. 2017).

The next step of the process is retrieval and recovery of polyhydroxyalkanoates. There are a few routes to recover the polyhydroxyalkanoates granules, including polyhydroxyalkanoates solubilization and the non-polyhydroxyalkanoates material dissolution (Visakh 2014), which are the main two routes employed. Non-polyhydroxyalkanoates dissolution step starts with the modification of the cell wall permeability, followed by the dissolution of the polyhydroxyalkanoates in a solvent, and finally solvent extraction. The solvent extraction methods are the most developed and most employed for polyhydroxyalkanoates polymer recovery as they result in a high purity of polyhydroxyalkanoates. Solvents such as chloroform, methylene chloride, and 1,2-dichloroethane were employed for polyhydroxybutyrate extraction and recovery (Tan et al. 2014). However, the issue with the non-polyhydroxyalkanoates material dissolution method is the high costs and solvent disposal when employed at a large scale as the amount of solvent needed is high (Chee et al. 2010; Visakh 2014). Subsequently, the solubilization method was

explored, which consists of mixing the polyhydroxyalkanoates-containing cells in an immiscible solvent with water at high temperatures. Then, the polyhydroxyalkanoates are extracted by the addition of cold water. The advantage of this method is the ability to recycle the solvent a number of times before disposing of it (Visakh 2014).

To overcome the drawbacks of the solvent extraction method, the digestion method was explored as a slightly more environmentally friendly alternative. Sodium hypochlorite is used to dissolve non-polyhydroxyalkanoates biomass followed by centrifugation to recover polyhydroxybutyrate polymer. However, this method can lower the polyhydroxyalkanoates content (Tan et al. 2014). To overcome this issue, an additional chemical, chloroform, is added to the solution to protect the polyhydroxyalkanoates from degradation (Visakh 2014; Tan et al. 2014). The solvent is then extracted and recovered via filtration. Other methods were developed to enhance the mentioned routes such as solid shear (Visakh 2014) or enzymatic digestion (Tan et al. 2014).

Enzymatic digestion was found to produce better results as it causes negligible degradation of polyhydroxyalkanoates produced. It consists of enzymatic hydrolysis of the cells first, followed by polyhydroxyalkanoates recovery by centrifugation. It was reported that polyhydroxyalkanoates with a purity of 90% were achieved via the enzyme digestion method (Tan et al. 2014). Although this method is safer compared to its counterparts and has lower environmental footprint, its expensive price increases the overall polyhydroxyalkanoates costs for a lower purity. More recently, new methods have been explored for the recovery of polyhydroxyalkanoates including supercritical fluid disruption (Hejazi et al. 2003) and dissolved-air flotation (Van Hee et al. 2006). Hejazi et al. were able to recover polyhydroxyalkanoates by the disturbing *Cupriavidus necator* cells by the pressurization of a supercritical fluid (CO_2) to the environment and increasing the temperature and pressure of the system over the critical point of the supercritical fluid. After a period of time, the pressure was released causing a pressure drop that disrupted the cells (Hejazi et al. 2003). For the dissolved-air flotation recovery, Van Hee et al. diluted the fermentation broth before putting it in a flotation column, then the bottom phase was freeze-dried to recover polyhydroxyalkanoates granules (Van Hee et al. 2006) (Table 5.5).

The fourth step involves the accumulation of polyhydroxyalkanoates via precipitation, centrifugation, or filtration in order to concentrate the polyhydroxyalkanoates and remove the cellular debris (Kourmentza et al. 2017). The solution is subjected to rotary evaporation followed by precipitation of polyhydroxyalkanoates due to the addition of ice-cold ethanol or methanol depending on the chain length (Tan et al. 2014). The last and final step is the purification, which involves processes such as washing, drying, and polishing (Kourmentza et al. 2017).

A suitable polyhydroxyalkanoates extraction route selection depends on various factors such as reaction time, pH and concentration, and recyclability of chemicals. However, in order to reduce the costs of polyhydroxyalkanoates production and environmental burdens, a system with a non-solvent harvesting recovery method is the most beneficial (Visakh 2014).

Table 5.5 Advantages and disadvantages of some polyhydroxyalkanoates recovery methods (Chee et al. 2010; Hejazi et al. 2003)

Recovery method	Advantages	Disadvantages
Polyhydroxyalkanoates solubilization	Simple High purity Recyclable Little to no degradation of polyhydroxyalkanoates High molecular weight recovered	Not environmentally friendly Large amounts of solvent required Long process
Non-polyhydroxyalkanoates material dissolution		Use of toxic solvents Costly Low yield of polyhydroxyalkanoates
Digestion	Slightly more environmentally friendly Effective for non-polyhydroxyalkanoates digestion	Severe degradation of polyhydroxyalkanoates Lower polyhydroxyalkanoates recovery
Enzymatic digestion	Negligible degradation of polyhydroxyalkanoates Most environmentally friendly Highest recovery of 90%	Expensive method Selective separation Complex process Heat shock treatment required as precursor
Dissolved-air flotation	No chemicals involved Little contamination	Floating step needs to be repeated Requires extraction first
Supercritical fluid (Van Hee et al. 2006)	Simple Cheap Rapid Environmentally friendly	Dependent on process parameters Frequent cleanup

5.9 Polyhydroxyalkanoates Applications

Polyhydroxyalkanoates are biodegradable plastics that are water-resistant and stable in air, which make them a promising substitute for petroleum-based plastics, especially for single-use items. It is estimated that polyhydroxyalkanoates can substitute up to 33% of the current commercial polymers (Castilho et al. 2009). Packaging is the considered the biggest industry for use of the polymer, especially for food packaging (Bugnicourt et al. 2014). The most frequently used polyhydroxyalkanoates are polyhydroxybutyrate and polyhydroxybutyrate-co-valerate as they are the most readily formed and present a convenient and flexible option for packaging, especially thermal flexibility (Visakh 2014). Properties of polyhydroxyalkanoates include being water insoluble, resistant to water degradation and UV, as well as sinking in water (Bugnicourt et al. 2014).

In the medical field, polyhydroxyalkanoates have a few advantages over silicone (Chee et al. 2010). First, polyhydroxyalkanoates are inert and degrade very slowly

inside the human body (Verlinden et al. 2007; Li et al. 2016), which are useful properties for delayed release of drugs (Rodriguez-Valera 1991). The hydroxybutyrate monomer is a blood constituent, and therefore, polyhydroxybutyrate can be potentially used in medical applications such as wound management and vascular system devices (Castilho et al. 2009). It is also explored for potential use with anti-HIV drugs and antibiotics thanks to its biocompatibility (Tan et al. 2014). It has been used to produce tissue scaffolds as well as artificial heart valves (Lutke-Eversloh and Steinbuchel 2004). Moreover, thanks to its biocompatibility, polyhydroxyalkanoates are a suitable component for skincare products (Chee et al. 2010) and personal hygiene products such as diapers (Verlinden et al. 2007). Polyhydroxyalkanoates have already been used in few packaging applications such as shampoo bottles, plastic bags, feminine hygiene products, carpets, and others (Dietrich et al. 2017). More recently, polyhydroxyalkanoates were used in toners for printers and in adhesives (Verlinden et al. 2007).

There are more future possible applications for polyhydroxyalkanoates that are currently under consideration. For instance, polyhydroxyalkanoates were explored as an option for the production of wood plastic composites where the petroleum-based matrix is replaced by polyhydroxyalkanoates to produce biodegradable wood plastic composites (Vandi et al. 2018). In the agricultural field, polyhydroxyalkanoates could potentially be used for the encapsulation of fertilizers and seeds to delay the release as they are biodegradable (Verlinden et al. 2007). However, one of the limitations of polyhydroxyalkanoates resides in their low resistance toward acids and bases, which can make them dissolve (Bugnicourt et al. 2014).

5.10 Sustainability Assessment

The premise of moving to bioplastics is that they can help conserve fossil resources by reducing their use while at the same time reducing emissions of CO_2 (Bugnicourt et al. 2014) and accumulation of nonbiodegradable plastics in the environment. There are two main approaches to evaluate the impact of polyhydroxyalkanoates production. Techno-economic analysis, which considers the economic performance of the process, and sustainability assessment, which evaluates the environmental and social performance (Dietrich et al. 2017). In order to evaluate the environmental impact, different tools can be employed depending on various aspects of sustainability. Life cycle analysis (LCA) is the most widely used assessment tool as it evaluates the environmental impact of the entire production process, starting from biomass collection to the end use of the product. Another popular tool is the sustainable process index where the entire chain of polyhydroxyalkanoates production is measured.

Based on conducted LCAs, the production of polyhydroxyalkanoates by microbial mixed cultures treating industrial wastewaters is more advantageous compared to pure cultures fed agricultural feedstocks, both environmentally and economically (Dacosta et al. 2015; Gurieff and Lant 2007). A significant portion of these benefits

is associated with the feedstock. From an economic perspective, agricultural feedstocks may make up around 25% of the total production cost. In comparison, use of industrial wastewater allows offset of treatment costs or trade waste tariffs, which can result in 25% cost reductions (Dacosta et al. 2015, Gurieff and Lant 2007). From an environmental perspective, most life cycle analysis studies show polyhydroxyalkanoates to be more environmentally friendly than petrochemical plastics (Leong et al. 2016) or at least similar (Hottle et al. 2013). However, a few studies have demonstrated that pure culture polyhydroxyalkanoates production from agricultural feedstock may be worse than petrochemical plastics, primarily due to the impacts associated with crop production and sterilization (Gerngross 1999; Kim and Dale 2005). Combined industrial wastewater treatment and polyhydroxyalkanoates production avoids issues with both crop production and sterilization. However, while Dacosta et al. (Dacosta et al. 2015) showed that GHG emissions were slightly lower than pure culture agricultural feedstock polyhydroxyalkanoates or petrochemical PET, nonrenewable energy use was significantly higher than both alternatives. High energy use was similarly reported by Gurieff and Lant (2007) and Gerngross (1999). However, replacement of energy sources with renewables provides a means to significantly reduce these impacts (Kim and Dale 2005). Moreover, microbial-based polyhydroxyalkanoates production has been shown to be significantly more environmentally friendly than plant-based polyhydroxyalkanoates production, mostly due to the high steam requirements in processing plant material (Zhong et al. 2009).

Areas of improvement in the process could lead to significantly reduced environmental footprint of polyhydroxyalkanoates production. One area of potential focus is the solvent extraction, which is a major contributor of overall impacts in an industrial wastewater treatment polyhydroxyalkanoates production facility (Dacosta et al. 2015). The use of alternative solvents is one approach. For instance, a comparison of various implementations of dimethyl carbonate as solvent has shown benefits across all assessed impact categories in comparison to a more traditional 1,2-dichloroethane solvent followed by methanol precipitation (Righi et al. 2017). Koller and Braunegg (Martin and Gerhart 2018) also highlight the need for fully integrated plant design and the benefits achieved when making use of harvested cell debris for biogas production and the subsequent supernatant as a nutrient source to the process. Fermentation nutrient addition (notably nitrogen) and aeration are two significant factors leading to environmental burdens from microbial mixed culture–based polyhydroxyalkanoates (Gerngross 1999), and the ability to reduce these through smart process design has potential for reduced impacts.

While a number of studies exist for assessing environmental impacts of polyhydroxyalkanoates, only a handful of these are related to microbial mixed cultures and integration with wastewater treatment. These studies have focused on similar process layouts consisting of three-stage fermentation and hypochlorite/chlorine-based cell digestion for extraction (Gurieff and Lant 2007; Dacosta et al. 2015). Therefore, there is a need for further studies in this area, particularly exploring different fermentation and extraction techniques due to the significantly lower biomass concentrations that may impact assessment results in comparison to pure culture systems.

Moreover, the majority of polyhydroxyalkanoate production studies have focused on cradle-to-gate ignoring use and end of life disposal. Given that one major driver for polyhydroxyalkanoates is its biodegradability, there is a need for analyses to encompass this important aspect. However, to date marine biodiversity loss due to plastic debris is a missing component from life cycle analysis methodology due to lack of sufficient data on impacts, degradation, and transport/fate routes (Woods et al. 2016). In contrast, end of life impacts for greenhouse gas emissions may not be positive, with Hottle et al. (2013) demonstrating high GHGs associated with methane emissions from disposal of biopolymers in landfills, for example. However, end of life disposal of biopolymers is currently with a high degree of uncertainty, and with appropriate end of life disposal routes such as anaerobic digestion or landfill with dedicated methane capture, the benefits should be positive.

Economics are another critical aspect to the sustainability and uptake of this technology. Studies on industrial wastewater treatment coupled with polyhydroxyalkanoates place the cost of produced polyhydroxyalkanoates in the order of $2–3/kg (Hassan et al. 1997; Gurieff and Lant 2007; Dacosta et al. 2015) which is significantly less than the current market prices for polyhydroxyalkanoates of approximately $5/kg (Kourmentza et al. 2017; Fradinho et al. 2014). Costs of production appear most reliant on polyhydroxyalkanoates extraction due to the high cost of solvents (Hassan et al. 1997; Dacosta et al. 2015). This aspect accounted for around three quarters of the total production price in the study of Hassan et al. using a photosynthetic-based open pond systems for treatment of palm oil mill effluent. The same authors demonstrated that polyhydroxyalkanoates accumulation and size of plant had relatively minor impacts on the production cost, although Gurieff and Lant (2007) indicated plant size and substrate concentration were much more important with their heterotrophic three-stage reactor system. However, economic analyses to date have been based on laboratory and small pilot-scale studies, and there is a need for costing and optimization based on data from development of full-scale systems that are not yet developed for microbial mixed cultures.

5.11 Conclusion

Polyhydroxyalkanoates are valuable biopolymer due to its ease of production from a variety of feedstocks, its inherent biodegradability, as well as a variety of other characteristics including UV resistance and limited oxygen permeability. Under increasing actions globally to mitigate climate change through reduced reliance on fossil fuels and efforts to reduce environmental burdens of plastic waste, there is a growing market for polyhydroxyalkanoates and other biopolymers. Correspondingly, there is an increasing trend of viewing wastewater treatment plants as resource recovery factories rather than simple pollution control facilities. These developments open up the opportunity for incorporation of polyhydroxyalkanoates production into existing industrial wastewater plants in order to recover the abundant chemical energy present.

The high cost of polyhydroxyalkanoates production compared with petrochemical plastics has limited polyhydroxyalkanoates development to date with only a handful of commercial facilities in operation (Kourmentza et al. 2017). Industrial wastewaters are typically high strength organic laden and nutrient deficient making them highly suitable for polyhydroxyalkanoates feedstock. Moreover, their simultaneous treatment allows cost offsetting unlike typical commercial agroindustry feedstocks which represent a significant proportion of the overall polyhydroxyalkanoates production cost. However, due to the non-sterile nature of industrial wastewaters and the typically open treatment systems employed at industrial facilities, microbial mixed cultures rather than pure cultures are more suitable. Microbial mixed cultures provide new challenges with respect to maintaining microbial communities with high polyhydroxyalkanoates accumulation rates and yields, as well as their downstream processing for polyhydroxyalkanoates extraction due to lower biomass concentrations. Currently, microbial mixed culture systems applied to municipal and industrial wastewaters only exist at pilot scale, and further research and development is needed to see commercialization (Kourmentza et al. 2017). Moreover, due to the high capital investment needed, government policy and incentives may be necessary to see the necessary paradigm shift in wastewater handling. Nevertheless, existing techno-economic and sustainability evaluations of microbial mixed cultures for combined industrial wastewater treatment and polyhydroxyalkanoates production provide promising indicators for implementation and commercialization (Gurieff and Lant 2007; Dacosta et al. 2015).

The major bottlenecks of the existing process include increasing biomass concentrations to simplify downstream processing, reducing energy costs associated with aeration and polymer extraction, and developing more sustainable extraction processes that simultaneously provide high purity and recovery. Significant developments have been made in recent years on fermentation techniques to overcome these constraints and promote higher polyhydroxyalkanoates accumulation, yields, and production rates and to produce selected monomers for desired properties. Such techniques include various substrate and nutrient feeding strategies, optimized fermentation and volatile fatty acid selection, and various novel selection strategies, including photosynthetic systems, settling selective pressures, and halophile cultures. The large variation in wastewater characteristics from industry to industry makes this a vast area for further research and innovation, and recent microbial mixed culture pilot-scale systems treating wastewaters (Morgan-Sagastume et al. 2015; Bengtsson et al. 2017) indicate near readiness of the technology.

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Chapter 6
Fouling and Wetting: A Major Challenge for Membrane Distillation

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Contents

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Abstract Membrane distillation is a widely adapted thermally driven technology that offers versatilities in desalination and wastewater treatment. The prominent challenge for membrane distillation is membrane fouling and wetting that has received amplitude of attention over the past few decades, but very few researchers have explored the minimization of fouling-wetting phenomenon so far. This book chapter specifically focusses on mechanism of fouling and wetting, achievable prevention techniques, and selection of key parameters of membrane properties, useful in curtailing the concerns of membrane fouling. Even the role of hydrophobicity, pore size, and thickness of membrane is discussed in this article. The book chapter also illustrates the stages of membrane fouling and its harmful effects, fouling differences due to various sources of water, effects of operating parameters, fouling prevention, and cleaning techniques. The mechanism of wetting along with degree of wetting has been demonstrated thoroughly. Basically, this chapter provides a detailed insight of various foulants along with fouling mechanisms in membrane distillation process. The book chapter highlights a number of approaches on membrane fouling and wetting minimization including the pre-treatment techniques, optimization of operational conditions, and utilization of superhydrophobic membrane in membrane distillation process.

Keywords Membrane distillation · Membrane fouling · Scaling · Wetting · Superhydrophobic membrane · Cleaning techniques · Anti-wetting · Wastewater treatment

Nomenclature and Abbreviations

FO	Forward osmosis
MD	Membrane distillation
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
PP	Polypropylene
DS	Draw solution
FS	Feed solution
NOM	Natural organic matters
RO	Reverse osmosis
NF	Nanofiltration
LEP	Liquid entry pressure
MWCO	Molecular weight cutoff
J_w	Water flux (L/m^2 h)
R%	Salt rejection %

6.1 Introduction

Good water and its availability are the key features contributing to habitable ecosystem, acceptable human health, and admirable socioeconomic development. However, inflated population, poor sanitation, rapid industrialization, and limited environmental concerns have led to the degradation of water cycle. It is estimated that 80% of the wastewater merges into the ecosystem without any pre-treatment or being reused (UNESCO 2017). UNDESA (2014) states that by 2050, about 70% of the world's population will live in cities (Barnett et al. 2005; Hoekstra et al. 2012; Purkait and Singh 2018; Rockström et al. 2009; Singh and Purkait 2019; Yadav et al. 2014); most of the cities lack requisite infrastructure and resources to address wastewater management in an efficient and sustainable way. With the ever-growing population and degraded ecosystem, ensuring safe and sufficient water supply becomes increasingly challenging. Thereby, a considerable solution to provide less polluting and enhanced techniques for wastewater treatment is of global importance.

Although a wide variety of techniques including adsorption, advanced oxidation, ion exchange, and activated sludge processes have been used, membrane treatment technology has progressively remained prominent in the wastewater industry. Among the membrane treatment technologies, reverse osmosis (RO), forward osmosis (FO), membrane filtration (MF), nanofiltration (NF), and ultrafiltration (UF) provide a conservative, economical, scalable, and comparatively simpler treatment strategies (Khawaji et al. 2008; Masafu et al. 2016; Ray et al. 2018c). To the contrary, membrane distillation has been less adopted due to its complex and formidable synthesis techniques. The process results in sequestration of the target compound(s) based on membrane-contaminant surface interaction, usually with the help of a porous membrane with good selectivity for the extract. Additionally, in the non-isothermal separation technique, vapors are transported (thermally driven) through a non-wetted hydrophobic membrane. The propulsive force of the process is the difference of vapor pressure between the two sides of the membrane pores accompanied by heat and mass transfer phenomena through the membrane (Alkhudhiri et al. 2012; Lawson and Lloyd 1997; Martínez-Díez and Vazquez-Gonzalez 1999; Yadav et al. 2014).

Although membrane distillation (MD) has shown promising results in water treatment, the evidences of membrane fouling and wetting are major concerns. Membrane fouling in a simplistic approach can be described as the accumulation of the foulants (dissolved materials and particles) into the pores of membrane, while the feed stream passes through the membrane during filtration. Consequently, the decrease in water flux at constant operating parameters indicates the phase of membrane fouling. In general, the colloidal foulants interact among each other, followed by membrane surface interaction to form depositions. Fouling formation mechanism can be understood by examining the forces of interaction between the particles (foulants) and the membrane surface (Gryta 2008b; Tijing et al. 2015). Correlating to Mayer et al. (2006), membrane fouling is very evident in membrane distillation, for a hydrophobic membrane is used to treat feed with abundance of hydrophobic contaminants (Mayer et al. 2006). Similarly, material of construction, membrane

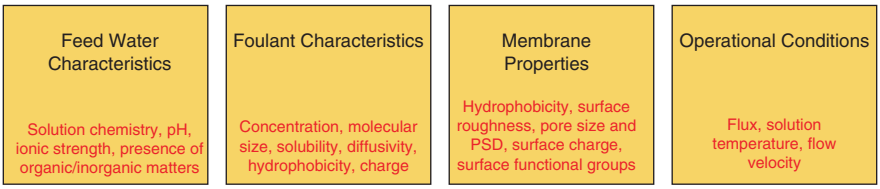

Fig. 6.1 Factors contributing to membrane fouling: (**a**) feed stream solution characteristics (chemistry, pH of solution, ionic strength, presence of various materials), (**b**) characteristics of foulant (concentration, molecular size, solubility, diffusivity, hydrophobicity, charge, etc.), (**c**) membrane features (hydrophobicity, hydrophilicity, surface roughness, pore size and pore size distribution, surface charge, surface functional chemical groups), and (**d**) operational parameters (water flux, temperature, flow speed, cross flow rate). PSD: pore size distribution

characteristics, operational parameters, nature of feed, and prominently the pore size of the membrane contribute to fouling. Typically, types of membrane, performance, hydrodynamic conditions, and operating conditions are prominent factors that influence the performance (Al-Amoudi 2010; Bellona et al. 2004; Camacho et al. 2013; J Zhang et al. 2006). Figure 6.1 shows the summary of all factors that affect the membrane fouling which include feed water characteristics, foulant characteristics, membrane properties, and operational conditions.

Prior to the details of membrane fouling, it is imperative to highlight the concept of fouling that can be sorted based on type of foulant, site of deposition of the foulants, and degree of deposition. For fouling to occur, the foulants can be organic, inorganic, and biological. Generally, the scaling over the surface of the membrane is inorganic foulants (mostly Ca^{+2}, Mg^{+2} salts) (Duong et al. 2015; Zhao and Zou 2011). On the other hand, as the name suggests, organic fouling has depositions, followed by coagulation of organic substances, quantified in the form of dissolved organic carbon. Similarly, algae and bacterial entities over the surface of the membrane form an extracellular polymeric substance that is viscous, slimy, hydrated, and form of gel.

Corresponding to investigations of Konieczny et al., the degree of deposition can be classified in terms of blocking as complete, standard, intermediate, and cake layer formation (Konieczny and Rafa 2000). When all the molecules of the suspension on the feed side block the pores, without subsiding (without influence) on each other, then complete blocking occurs. It is assumed that all molecules of the suspension take part in the blocking of pores. The mechanism of standard blocking of pores draws a proportionality correlation between the volume of the pore and the volume of the filtrate, assuming that the entire phenomenon occurs inside the pores. Transitory or intermediate blocking occurs only when the foulants subside over each other on the wall of the pores, although some of the foulants have settled on the membrane. The probability of foulants to reach the pores gets smaller and smaller as the blocking increases. Correspondingly, since the cake formation of foulants is only a surface phenomenon, perhaps it increases the surface resistance, implying a reduced flow rate of filtrate over the surface of the membrane (Gryta 2008b; Vrijenhoek et al. 2001; Warsinger et al. 2015).

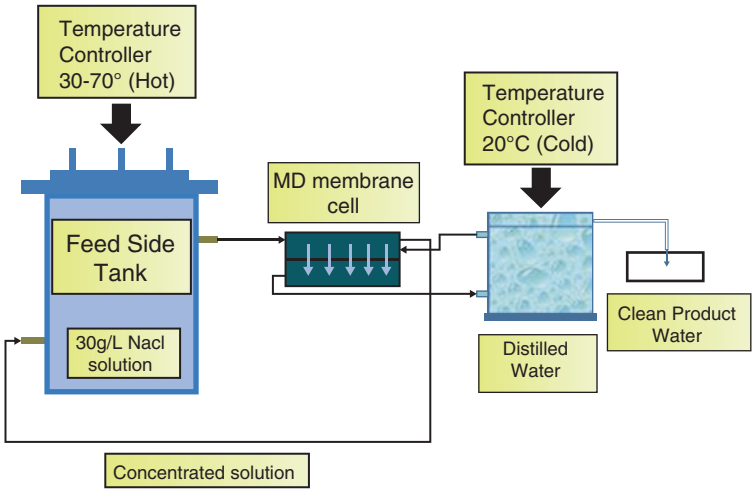

Fig. 6.2 A general diagrammatic representation of membrane distillation (MD). In case of membrane distillation, the difference of partial vapor pressure commonly initiated by difference in temperature is the contributing force. (Note: Adapted and modified from Ray et al. (2018c))

As mentioned earlier, membrane distillation is a thermal separation technique, wherein a hydrophobic microporous membrane isolates two aqueous streams at different temperatures. Correspondingly, an interface between gas and liquid is generated due to lack of mass transfer by virtue of its hydrophobic character (Ray et al. 2017a; Ray et al. 2019; Schofield et al. 1987). A typical schematic diagram is shown in Fig. 6.2 to illustrate the process of membrane distillation for desalination as well as water treatment.

Membrane distillation has two major problems: wetting and fouling. Fouling during membrane distillation can be broadly classified based on the deposition site of the foulants as internal fouling and external fouling. As the name suggests, external fouling is a surface phenomenon, wherein the foulants get accumulated on membrane surface (feed side). It is reversible in nature as the foulants can be simply washed off from the surface. To the contrary, internal fouling, commonly called pore blocking, wherein the pores are completely packed with foulants, is irreversible, implying a permanent damage to the membrane (Ge et al. 2014; Goh et al. 2013a). Another category of fouling exclusively observed in membrane distillation is membrane wetting (Goh et al. 2013b; Lu et al. 2008). It can be described as a phenomenon where the feed liquid occupies the pores, instead of the vapors. The aforementioned phenomenon occurs, corresponding to the increase in surface tension of the liquid feed at the interface of liquid-vapor on the surface, resulting the liquid to flow toward the larger pores of the membrane. As soon as the liquid pressure exceeds the critical penetration pressure (depends on pore size), it penetrates into the membrane pores. The wettability of membranes can also be classified as external and internal. In external wetting, the liquid is present only on the surface, whereas the pores inside the membrane walls are dry or partially wetted. On the

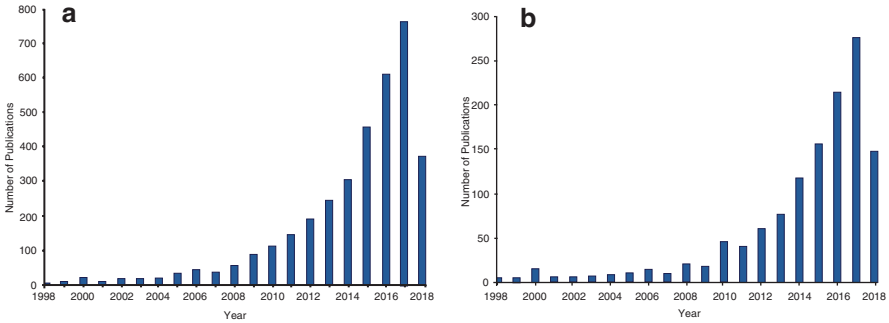

Fig. 6.3 (**a**) Observation of the number of publications (peer reviewed) since 1998 (Data analysis of publications was executed by utilizing the Scopus scholar search system with the term "membrane distillation" and "membrane fouling," as on September 2018.). (**b**) Observation of the number of publications (peer reviewed) since 1998. (Data analysis of publications was based on Scopus scholar search system with the term "membrane distillation" and "wetting," as on June 2018)

contrary, whenever the membrane walls inside the pores are completely wet, it can be classified as internal wetting (Gryta 2005; Lu et al. 2008).

Over the past decade, membrane distillation has gained much popularity, as a result of increasing versatility in water treatment and desalination. Nevertheless, membrane fouling and wetting emerge out as prominent issues related with membrane distillation process. A survey of peer-reviewed publications, pertaining to "membrane distillation" and "membrane fouling" of the last 20 years, is shown in Fig. 6.3 (a). Furthermore, Fig. 6.3 (b) shows the survey of peer-reviewed publications related to "membrane distillation" and "wetting" of the last two decades. This data collected from the Scopus-based advanced scholar search system significantly highlights the rise in research on membrane distillation processes and the advancement in efficient membrane technology.

Therefore, it becomes necessary to focus on membrane distillation techniques to explore its future prospective and development. This book chapter shows recent studies on membrane fouling in membrane distillation process, with more focus on reinforcement and post treatment.

6.2 Membrane Fouling Phenomena in Membrane Distillation

Over the past few decades, numerous studies have been performed on types and degree of fouling, but limited studies detailing the site of fouling have been performed. Conveniently, as mentioned in the introduction, fouling depending on the site of deposition can be classified as cake layer formation (external) and pore blocking (internal). As mentioned in the introduction section, pore blocking is

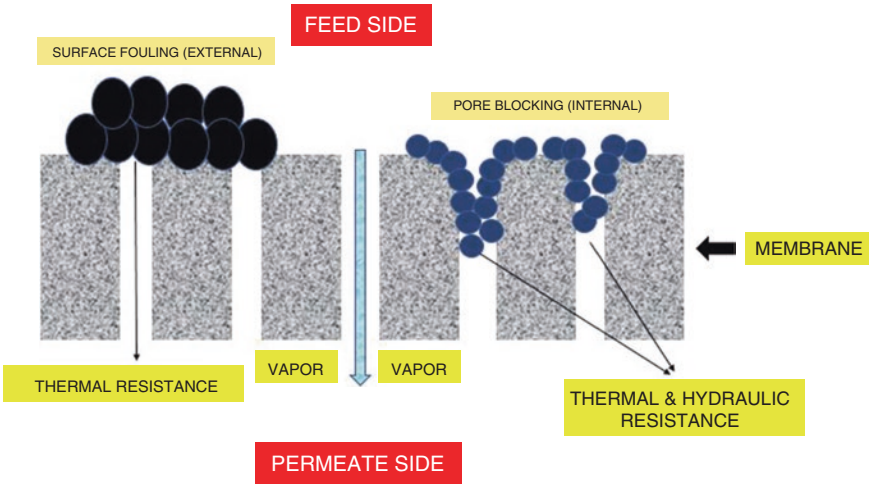

Fig. 6.4 The fouling sites on a membrane can be classified into external fouling (cake layer formation) and internal fouling (pore blocking) depending on nature and composition of feed stream solution

irreversible in nature, whereas cake layer formation is reversible in nature, considering the compactness of the foulants. In most cases, that implies to a permanent damage to the membrane (Tijing et al. 2015). Figure 6.4 shows the typical fouling sites on a membrane that may cause thermal as well as hydraulic resistance.

6.2.1 Pore Clogging

Pore clogging is also termed as internal fouling (complete blocking, standard blocking, and intermediate blocking) that happens when foulants clog the pores of the membrane. Of both the types of fouling, internal and external, investigations have reported that internal fouling precedes external fouling, although in some cases a cake layer is formed that acts as a secondary layer or membrane (Srisurichan et al. 2005). According to Shirazi et al., most of the pore blocking take place in inorganic fouling due to the deposition of the salts (Shirazi et al. 2010). Although it is considered that membrane distillation happens to have the least degree of fouling, relating to the larger pore size, pore clogging reduces the pore volume and surface area. Consequently, the reduced pore volume decelerates the water vaporization and decreases the permeate flow rate. Additionally, these hindrances reduce the area of flow, escalating the pressure drop with limited flow rate, contributing to a higher temperature of polarization and reduced flux.

However, from the optimization studies performed by Jiang et al., it was evident that at a lower feed rates, pore blocking was higher than cake layer formation

and was the most contributing mechanism for fouling (Jiang et al. 2005, 2003). However, increase in cake layer formation was observed with a rise in feed rate. Later, under the same investigation, the increase in pore blocking with the decrease in temperature was confirmed. Also, pore blocking is highly dependent on the type of polymer used for membrane fabrication. Similar to studies by Gryta et al., influencing the membrane performance, it was observed that the gas retained in the membrane pores was a major factor contributing to the fouling internally (Gryta 2005), commonly observed in highly hydrophobic-polymeric membranes, viz., polyvinylidene fluoride (PVDF), polypropylene (PP), or polytetrafluoroethylene (PTFE). Correspondingly, the pressure on the liquid surface, the pore diameter, and the hydraulic pressure contribute to the penetration and retention of liquid inside the pores, described by the Laplace-Young equation (Kelvin law) (eq. (6.1)):

$$dp = PF - PD = \frac{-4B\sigma \cos\theta}{dp} \tag{6.1}$$

where B is the geometry coefficient (B = 1 for cylindrical pores), σ is the surface tension of the liquid, Θ is the contact angle, d_P is the pore diameter, and PF and PD are the hydraulic pressure on the feed and distillate side, respectively. Furthermore, with possibilities of membrane wetting, the maximum membrane pore diameter should not exceed 0.5 micrometers for membrane distillation process (Burgoyne and Vahdati 2000).

The following graphical presentation in Fig. 6.5 highlights the pore-blocking mechanism elaborately.

Although initially the permeate flux decreases rapidly due to the blockage of membrane pores, the maximum permeate flux can be observed for clean and unoccupied pores at the beginning. Subsequently, the permeate flux declines as the externalities block the membrane pores as pores of membrane are blocked by retained particulates/matters. Though the occupancy of membrane pores is a measure of relative size of particles, shape, and pores, the pore blockage is dominant with identical size and shape of particles and pores. When compared to cake layer formation, pore blockage is rapid as lesser number of particles is required for the occupancy (Guo et al. 2012), whereas water flux declines after pore blocking due to formation of cake layer on the surface of membrane.

6.2.2 Cake Layer Formation

Bacterial growth and scaling (for the high-concentration solution) contribute to the formation of an additional layer onto the surface of the membrane, consisting of the impurities present in the feed. Additionally, fouling and scaling on surfaces minimize the flow channel area which causes a pressure reduction and decreased flow

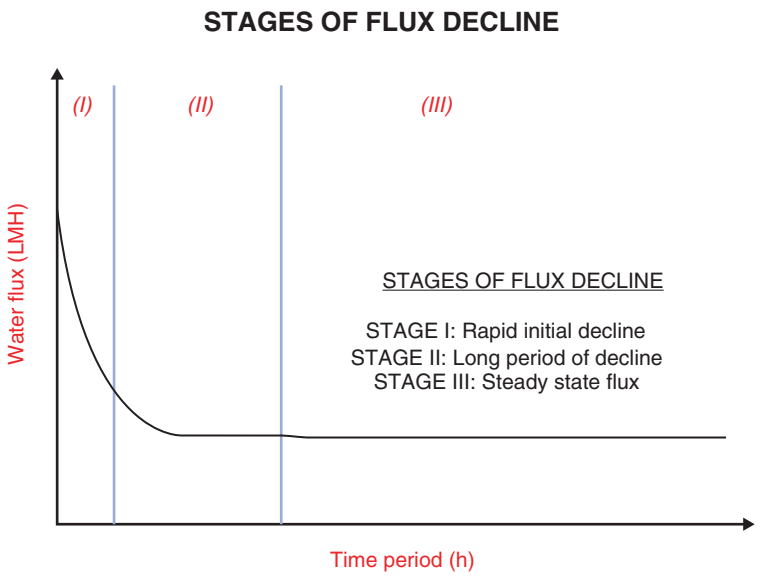

Fig. 6.5 Water flux-time plot for filtration process starts with (I) a rapid initial decrease of the water flux, (II) followed by a long period of gradual flux decline, and (III) ends with a steady-state flux

velocity, resulting in higher temperature polarization effects and lowering of permeate flux. Additionally, Gryta et al. noted the pore blockage of adjacent pores as a surface deposition (partial membrane wetting) (Gryta 2008a). Furthermore, this additional fouling layer increases the thermal resistance of the surface. External surface fouling is usually reversible and can be cleaned by physical or chemical cleaning. To explain the process of cake layer formation, it is very necessary to describe the contributing forces as well as operation conditions. However, the foulants also have a deciding role for determining the type of fouling to occur. Mostly, the foulants retained on the membrane and attach themselves on the membrane surface and contribute to cake layer growth, hereby increasing the resistance over the surface as R_{cake}, wherein the smaller particulates contribute more to the specific resistance of a filter cake. The aforementioned relation could be well explained by mathematical formula given by Blake-Kozeny equation (eq. 6.2) (Broeckmann et al. 2006; Huisman et al. 1998):

$$\frac{1}{K*} = K*cake = \frac{k\,Kozeny \times 90}{(dp*)^2} \cdot \frac{\left(\frac{Ccake}{\rho p}\right)^2}{\left(1-\frac{Ccake}{\rho p}\right)^3} \tag{6.2}$$

wherein d_p is the mean particle diameter, c_{cake} is the mean mass concentration of the foulants forming the cake layer, and ρ_p refers to the density of the particles. K* cake is the specific cake layer resistance. From the Blake-Kozeny equation, the inverse relation of the mean particle diameter and the specific resistance was confirmed. Hereby, it can be easily stated that smaller particles contribute much toward the cake layer formation as compared to larger foulants. Other than the size of the foulants, the cross flow rate also has significance on the deposition of the foulants on the membrane layer for cake layer formation. Recently, Mamun et al. projected the aggravated initial growth rate of the secondary layer corresponding to the escalated cross flow velocity (Chang et al. 2018).

According to the classic Derjaguin, Landau, Vervey, and Overbeek (DLVO) theory, the net foulant surface interaction additively depends on the van der Waals and electrical forces between the double layers (Hoek et al. 2003). The surface and the foulants with opposite charges will facilitate the cake layer formation, and correspondingly, similar charges destabilize the formation mechanism. From the aforementioned theory, it is very evident that particles with repulsive or lowered interaction with surface constitute to less fouling. Other than the surface-particle interaction, particle-particle interaction plays an acute role to enhance the cake layer formation. According to Wilkinson et al., for particles with higher frequency of collision and attachment coefficient, more foulants agglomerate on the surface (Wilkinson and Lead 2007). Overall, external layer adds to the thermal and hydraulic resistances, characterized by the porosity and thickness of the layer. As a result, the overall heat transfer coefficient is changed. Thus, Fig. 6.6 indicates the change in the thermal properties due to the formation of cake layer that decreases the temperature differences across the membrane and results lower water flux.

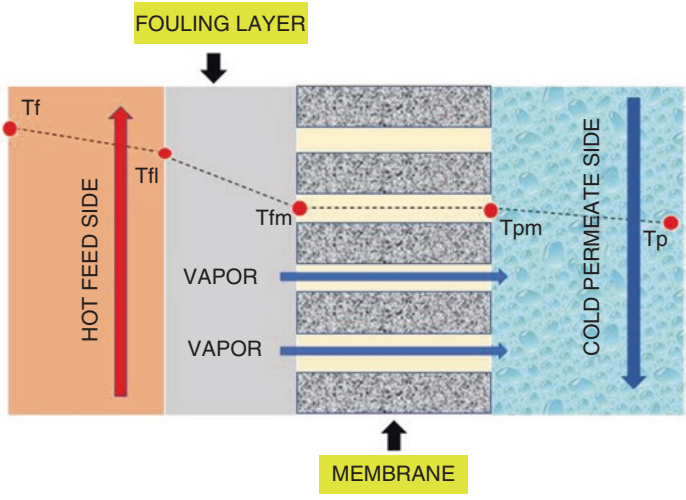

Fig. 6.6 Transition in thermal properties due to formation of the fouling layer which minimizes the temperature differences across the membrane and results in water flux decline

The fouling layer formed changes the thermal properties, minimizes the temperature difference across the membrane, and correspondingly increases the polarization temperature implying a lesser driving force (Hsu et al. 2002). One of the most important layer characteristic contributing to the resistances is the porosity. The secondary layer formed can be either porous or nonporous. Gryta explained the formation of porous layer by the deposition of smaller crystals due to higher velocity, while thicker layers ("mountain-like" structures) are formed with lower velocity (Gryta 2005). Although a nonporous layer increases both thermal and hydraulic resistance, the porous layer only effects the thermal resistance. Furthermore, less transport of water vapor and increased mass transfer resistance are observed for nonporous layer. Similarly, the escalated thermal resistance reduces the permeate flux for a porous cake layer (Gryta 2008b).

6.2.3 Particulate or Colloidal Fouling

Particulate or colloidal fouling is a common concern in all filtration technologies. Although the larger particles can be separated by advanced filtration (i.e., ultrafiltration, microfiltration, nanofiltration), the smaller particle still poses complications in membrane-based technologies. Generally, for pilot-scale membrane distillation, desalination units, cartridge filters, or screens are installed for efficient pre-treatment of particulate matter. These particulates are generally clay, humic substances, silt, and silica, abundant in ponds, lakes, groundwater, and especially river water, compared to seawater (Meng et al. 2014). Silica being small in size is harder to remove with pre-treatment stems such as microfiltration, contributing to a significant concern of silica fouling. Primarily, there are three forms of silica present in water supplies: colloidal silica, particulate silica, and dissolved silica (or monosilicic acid). Although recent investigation notes silica fouling to be predominant in membrane distillation systems, the flux decline is lesser than scaling. Furthermore, acid treatment to counter scaling is inefficient to remove the uncharged silica foulants (Drioli et al. 2015; Gilron et al. 2013).

6.2.4 Organic Fouling and Biofouling

Most of the recent research articles discussed about inorganic fouling as well as scaling in membrane distillation process. There are limited sources that elaborate the mechanism of organic and biofouling in membrane distillation process. However, biofouling or microbial growth shows minimal impact in membrane distillation process due to high saline concentration and high-temperature condition utilized in membrane distillation operation. Based on various water and wastewater studies, organic fouling as well as biofouling can also be considered as one of the main fouling categories. Table 6.1 indicates the typical mechanism of type of foulant including organic fouling and biofouling.

Table 6.1 Mechanism of organic fouling and biofouling in membrane distillation operation due to presence of natural organic matters and microorganisms in water sources

Type of fouling	Foulant sources	Typical mechanism involves	References
Organic fouling	Presence of natural organic sources in water sources	Organic fouling occurs via adsorption of natural organic sources on the membrane that causes a layer of gel formation of macromolecular substances	Naidu et al. (2014) and Srisurichan et al. (2005)
Biofouling (biofilm formation)	Microbial growth includes fungi, algae, and other microorganisms in water sources	Biofouling takes place when there is a formation of biofilm on the membrane surface	Goh et al. (2013b) and Krivorot et al. (2011)

Franken et al. explained that presence of natural organic matters (NOM) in high concentration in the feed stream decreases the hydrophobicity of the membrane through adsorption that causes wetting of membrane (Franken et al. 1987). Eventually, deterioration of membrane contact angle and increase in organic content in permeate stream were observed. In other words, the rate of wetting purely depends on the type of foulant (based on characteristics of feed stream) (Naidu et al. 2016). Figure 6.7 indicates the membrane distillation fouling-wetting mechanism based on organic fouling, and it also elaborates the consequences.

6.2.5 Scaling Phenomenon

Scaling of membrane takes place when inorganic salts precipitate and accumulate on the surface of the membrane, thereby clogging the pores for vapor to diffuse through the membrane and resulting in decline of permeate flux. $CaCO_3$, $CaSO_4$, and silicates were analyzed to be the prominent cause of permeate flux decline while recovering reusable water from natural streams including seawater and brine water stream. Membrane scaling falls into the category of membrane fouling. Typically, there are two types of membrane scaling, namely, (a) inorganic scaling and (b) particulate or colloidal fouling. In general, it is observed that the pressure-driven reverse systems assist the formation of compact cake scales, unlike membrane distillation process, driven by temperature difference (Martinetti et al. 2009; Warsinger et al. 2015).

In the recent years, extensive investigations have been performed for inorganic scaling in membrane distillation processes. Typically, inorganic scaling in both reverse osmosis and membrane distillation processes is categorized into (a) alkaline scaling, (b) non-alkaline scaling, and (c) uncharged molecule scales. The alkaline salts (basic) when added through hydrolysis make the solution basic, correspondingly increasing the hydroxide ion concentration. In general, reducing the pH below 7 limits the scale formation by alkaline salts like calcium carbonate. Non-alkaline salts constitute of readily dissolving charged ions that do not alter the pH of water.

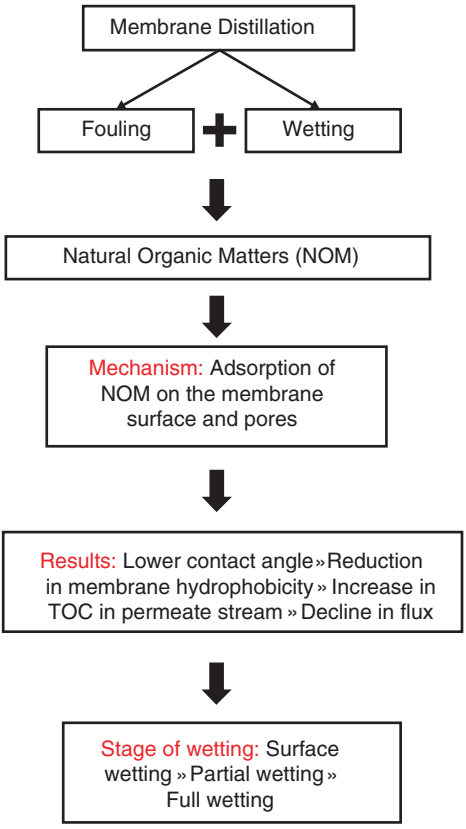

Fig. 6.7 Membrane distillation fouling and wetting tendency based on presence of natural organic matters (NOM) in feed stream that results in lower quality of permeate stream

Similarly, uncharged molecules like silica form scales. These lesser-soluble molecules dissociate highly polar water molecules to dissolve salts and lead to particulate scaling (Curcio et al. 2010). Among all scaling agents, calcium carbonate is one of the most common scales in membrane distillation desalination systems, further effecting the operating conditions in brackish, groundwater, and seawater desalination. Furthermore, $CaCO_3$ is a pervasive scale in both membrane distillation and reverse osmosis desalination that readily reaches supersaturated conditions followed by the breakdown of bicarbonate HCO_3^-, as shown in the equation below (Curcio et al. 2010):

$$Ca^2 + 2HCO_3 \rightarrow CaCO_3 + CO_2 + H_2O$$

Additionally, higher pH and carbonate concentration enhance scale formation in membrane distillation systems, proving the carbonate equilibrium and scaling to be complex processes. Recent investigations make it evident that this nonporous calcium carbonate scale in membrane distillation causes significant water flux decline.

6.3 Mechanism of Wetting in Membrane Distillation

Among many of the concerns related to efficient use of membrane distillation systems, the prominent challenge is membrane wetting, wherein product contamination is a consequence of saline water penetration through the membrane. However, there are numerous fouling mitigation methods, viz., superhydrophobicity, air backwashing to reverse or prevent wetting, non-fouling operating conditions, and maintaining air layers on the membrane surface. If a distillation membrane gets wetted and the micron-sized pores are flooded with liquid, the feed stream can easily pass through the flooded pores in its liquid form, failing the membrane as a barrier for salts, macromolecules, and other submicron size particles (Franken et al. 1987; Goh et al. 2013b). The wettability (S) of a material can be defined as the ability of a liquid to wet a surface. Additionally, the degree of wettability can also be referred as spreading which is controlled by interfacial forces set up between a solid (S), liquid (L), and vapor (V) phase at the minimum equilibrium distance. Typically, the interfacial forces are expressed as surface tensions (γ) and can be associated to the surface wettability coefficient by given mathematical eq. (6.3):

$$S = \gamma SV - \left(\gamma SL + \gamma LV \right) \tag{6.3}$$

There is another way to characterize the membrane wettability in terms of contact angle (θ), expressed mathematically by Young's equation (eq. (6.4)):

$$\cos\left(\theta\right) = \frac{\gamma SV - \gamma SL}{\gamma LV} \tag{6.4}$$

It is considered that (θ)= 90° as a borderline between hydrophobicity and hydrophilicity. Typically, contact angle lesser than 90° denotes hydrophilic surface with a positive liquid wetting; on the other hand, contact angle higher than 90° is considered as hydrophobic surface with negative wetting (Banat and Simandl 1994; Ge et al. 2014).

6.3.1 Degree of Wetting

Membrane wetting is a complex physicochemical process categorized into four phases (degrees) (Table 6.2): non-wetted (dry phase: 1st phase), surface wetted (2nd phase), partially wetted (3rd phase), and fully wetted (4th phase). Typically, in surface wetting, the interface of vapor/liquid shifts inward, toward the cross section of the membrane. Consequently, increasing the polarization temperature and decreasing the water flux reduce the temperature of the evaporating interface in the

Table 6.2 Degree of membrane wetting in membrane distillation operation: (a) first phase: non-wetted stage represents steady-state flux; (b) second phase: surface-wetted stage indicates drop in water flux; (c) third phase: partially wetted stage tends to fully wetted stage; and (d) fourth phase: fully wetted stage may decrease in product quality

Stages/ parameter	Stage I	Stage II	Stage III	Stage IV
Permeate flux	Steady-state flux	Drop in water flux as the surface gets wet	Increase in water flux	Flux increases due to least membrane barrier functionality leading to decrease in water quality
Rejection percentage	High rejection rate	Partial drop in rejection rate	Rejection rate continues to decrease	Least rejection rate
Initial membrane wetting	No wetting	Surface wetting	Partial wetting with a tendency to full wetting	Full wetting
Initial → **Final**				
Time duration				

membrane pore. Simultaneously, as the feed penetrates more into the pores, partial wetting occurs and lowers the water flux by limiting the active surface area for mass transport. Therefore, partial wetting results in deterioration of product quality. Unfortunately, for long-term operations, almost all the hydrophobic membranes used in membrane distillation (PTFE, PP, and PVDF) systems get contaminated by partial wetting. During full wetting, the membrane distillation process no longer remains as a barrier and leads to a viscous flow of liquid via pores, incapacitating the distillation process. Table 6.2 indicates qualitative analysis of water flux and rejection percentage for the membrane distillation operation based on the phase or degree of wetting (El-Bourawi et al. 2006).

6.3.2 Wetting Causes

The initial cause of the membrane wetting is fouling due to foulant deposition on the surface and pores of membrane. As mentioned earlier, the material of foulants categorizes fouling in membrane distillation systems. These include organic fouling such as biological fouling and particulate or colloidal fouling, as well as inorganic fouling or scaling. This deposition of materials can minimize liquid entry pressure (LEP), that is, the minimum pressure exerted by the liquid to enter the membrane pores, thus contributing to their hydrophobic nature. Furthermore, the depositions block the membrane pores and reduce the membrane efficiency by lowering the water flux and product quality (Gryta 2007). The numerous causes of wetting in membrane distillation are detailed in Table 6.3.

Table 6.3 Membrane wetting causes and mechanisms involved in membrane distillation process. Membrane degradation, organic fouling, membrane scaling, and transmembrane pressure are the prominent reasons of membrane wetting in membrane distillation

Type of cause	Mechanism involved	Reason
Membrane degradation (long-term operation)	Formation and deposition of hydrophilic chemical groups on membrane surface	Oxidative chemical or mechanical degradation
Organic fouling	Lowering of hydrophobicity of the membrane	(a) Attractive forces between hydrophobic materials within an aqueous system (b) Enhancing the affinity of membrane and solution
Membrane scaling	Lowering the hydrophobicity of membrane	Deposition on the membrane surface and crystallization inside the pores of membrane
Transmembrane pressure	Higher than liquid entry pressure	Pressure spikes, operating with low surface tension fluids or large pore size membrane

6.4 Minimization of Fouling and Wetting in Membrane Distillation Process

Although synthesis of membranes for distillation involves detailing and complexity, it has emerged as one of the most celebrated methods for water treatment. Over the recent decades, researchers have shown growing concern for the maintenance of the membranes and the mitigation methods of fouling, keeping the complexity of synthesis procedure in mind (G. Chen et al. 2013; Ding et al. 2011; Fane and Fell 1987; Ray et al. 2020). While prevention is the objective that can only be achieved at the laboratory scale, the reality offers reduction methods that can only be scaled up for industrial purposes. Some of the most prominent and efficient methods for fouling control are mentioned below: (a) conventional pre-treatment and (b) nonconventional treatment. Table 6.4 indicates the brief information collected from literature on the effectiveness of different cleaning techniques for restoring steady water flux. In general, after a long-term operation of membrane distillation, fouling and wetting may occur. Usually, backwash technique as well as drying can be effective to control fouling and wetting tendency. Eventually, physical cleaning or chemical cleaning will be required if the pervious technique doesn't work. In case the membrane distillation performance was not recovered, then it is recommended to replace the membrane. Hence, a typical protocol for controlling membrane fouling and wetting has been illustrated in Fig. 6.8 for better and stable performance.

Table 6.4 Cleaning techniques utilized in membrane distillation and reported flux recovery percentage. Typically, physical cleaning by water and mixture of acid are considered to be the effective cleaning techniques

Type of membranes	Feed stream	Cleaning technique	Permeate flux recovery percentage	References
Polytetrafluoroethylene (PTFE)	7 g/L NaCl	Washing by water	≈98	Pangarkar et al. (2011)
Polytetrafluoroethylene (PTFE)	Groundwater	Washing by water	≈94	Pangarkar et al. (2011)
Polypropylene (PP) hollow fiber	Groundwater ($CaCO_3$)	3 wt.% HCl	≈98	Gryta (2010)
Polypropylene (PP) hollow fiber	Tap water ($CaCO_3$)	3 wt.% HCl	≈98	Gryta (2010)
Polytetrafluoroethylene (PTFE)	Seawater	Piezoelectric transducer at 35 kHz utilized to induce cavitation and cleaning of membrane. Initially flux restored.	≈91	Hsu et al. (2002)
Polypropylene (PP) hollow fiber	Seawater (HA, $CaCO_3$)	20 min citric acid followed by 20 min NaOH two-stage cleaning	≈ 100	Curcio and Drioli (2005)
Polypropylene (PP) hollow fiber	Groundwater (primarily $CaCO_3$, $CaSO_4$)	2–5 wt.% HCl	≈ 100	Gryta (2008a)

6.4.1 Selection of Key Parameters of Membrane Properties

Membrane distillation (MD) was introduced for desalination in the 1960s as a separation process combining membrane and simultaneous evaporation. As earlier mentioned, membrane is used as a barrier that separates vapor and water from the bulk solution, and then, the vapor diffuses through the membrane pores and condenses at the permeate stream. According to the dynamics of membrane distillation, the process should meet the following criteria (Curcio and Drioli 2005). Table 6.5 indicates the prominent criteria of distillation membrane for better performance in terms of water flux and rejection.

Hydrophobicity

In membrane distillation process, the water cannot pass through the hydrophobic membrane. Nevertheless, at a certain pressure, the water goes into the membrane pore; this pressure is known as liquid entry pressure (LEP). Liquid entry pressure is considered to be one of the important properties of the membrane because it

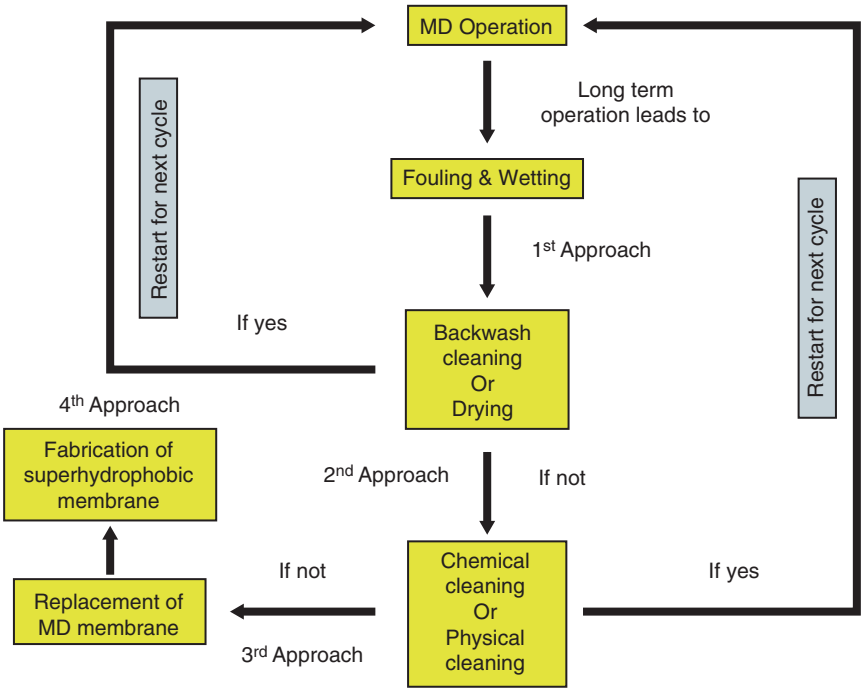

Fig. 6.8 Typical protocol for controlling fouling and wetting tendency in membrane distillation for long-term operation. The controlling techniques include backwash cleaning, drying of membrane, chemical and physical cleaning, and replacement with a new superhydrophobic membrane. MD: membrane distillation

Table 6.5 Criteria of distillation membrane for efficient performance. The basic requirement for ideal operation based on different properties has been mentioned

Properties	Basic requirement	References
Membrane thickness	*Optimum thickness:* Thickness was found to be inversely proportional to the mass and heat transfer rate across the membrane	Laganà et al. (2000) and Smolders and Franken (1989)
Pore distribution	*Narrow pore size:* Water cannot pass through the membrane pores and leads to wetting. The pore size of membrane ranges from few nanometers to few micrometers	Phattaranawik et al. (2003)
Liquid entry pressure (LEP)	*Higher liquid entry pressure:* If liquid entry pressure is low, this will result in membrane wetting that will affect the quality of permeate	García-Payo et al. (2000) and Ray et al. (2018)
Surface energy	*Low surface energy:* Corresponds to high hydrophobicity	Bonyadi and Chung (2007) and Jing Zhang et al. (2013)
Porosity	*Higher porosity:* Higher porosity allows more flux	Lawson and Lloyd (1997)

indicates the hydrophobicity of the distillation membrane. In general, membrane wetting can be evaluated by contact angle. Typically, higher contact angle, narrow pore size, and surface tension maximize liquid entry pressure. It is necessary to utilize distillation membranes with greater liquid entry pressure (LEP) value (Rácz et al. 2014). Franken et al. (Franken et al. 1987) have used a mathematical formula to evaluate LEP value which is based on Cantor-Laplace equation (Franken et al. 1987) (eq. 6.5):

$$\mathrm{LEP} = \frac{-2B\gamma L\cos\theta}{\mathrm{r\,max}} \tag{6.5}$$

where LEP indicates liquid entry pressure of water in Pa (Pascal), geometric factor is represented by B that is dimensionless and includes pore irregularities (B = 1 for assumed cylindrical pores), γL represents the liquid surface tension in N/m (in this case water at 25 °C, 0.07199 N/m), cos θ represents the contact angle in degree, and r_{max} indicates the maximal pore (non-closed) radius in meters (m).

Thus, to achieve higher liquid entry pressure (LEP), these following conditions should be improved:

- The membrane material should be hydrophobic or superhydrophobic in nature.
- The contact angle of the membrane should be higher.
- The membrane pore size should be narrow.

Typically, the contact angle (also known as wetting angle) is a measure of the wettability of a solid surface by a liquid. Contact angle (θ) can be defined as the angle where a liquid-vapor interface meets a solid surface, and this space includes liquid, vapor, and solid. The hydrophobicity and wetting of the membrane surface can be analyzed based on the contact angle of the membrane surface. Figure 6.9 indicates the

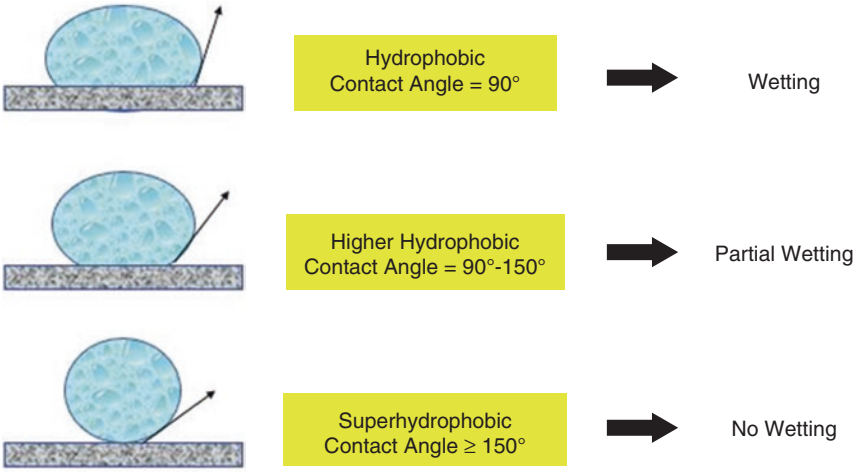

Fig. 6.9 Effect of contact angle on membrane wetting. Higher contact angle is preferable for achieving anti-wetting membrane in membrane distillation

effect of contact angle on membrane wetting. Furthermore, it also shows that when contact angle is higher than 150°, the chances of membrane wetting are apparently lesser as compared to the membrane possessing lower contact angle (Khayet et al. 2006).

Pore Size Distribution

In general, membrane pore size affects the water flux as well as membrane wetting, so it can be considered as one of the influencing factors of the membrane. Therefore, the selection of the membrane pore size should be carefully considered. However, there can be two cases: (a) if the pores were too large, the water easily passed through the membrane, which creates membrane wetting, and (b) if the membrane pores were too narrow, the vapor that permeates the membrane will be too less, and thus, the water flux will reduce. To make sure that the operating temperature and pressure change could not cause wetting of the membrane, it was recommended that the membrane pore size must be within the range of 0.1 and 1 μm (Kimura et al. 1987; Laganà et al. 2000).

Membrane Thickness

In general, it is believed that the membrane thickness must be optimized as it may influence the permeate flux and declined thermal resistance (tend to decrease heat efficiency or interface temperature difference) as the membrane thickness is getting lowered (Dommati et al. 2019; X. Zhang et al. 2011). As per eq. (6.6), lowering the membrane thickness cultivates the sensible heat loss from the hot feed stream to the cold permeate stream, further decreasing the water flux as a consequence of reduced interfacial temperature differences (vapor pressure difference). Therefore, it can be concluded that there should be an optimized membrane thickness for membrane distillation's effective performance (Camacho et al. 2013):

$$a\left(Tf - T1\right) = \frac{\delta}{b}\left(T1 - T2\right) + JHg \tag{6.6}$$

where T_f represents feed temperature, T_1 represents the temperature drop across the feed stream, T_2 represents the temperature of the membrane surface on the cold permeate stream, δ is the thermal conductivity of the membrane, b is the thickness of membrane, α is the convective heat transfer coefficient on the feed stream, J is the permeate water flux, and Hg is the enthalpy of the vapor. Therefore, many research works showed that effective membrane thickness for distillation process ranges from 60 to 200 μm (Ray, Chen, Nguyen, et al., 2017).

6.4.2 Thermal Condition in Membrane Distillation Operation

Temperature is one of the significant factors contributing to fouling and scaling in membrane distillation. Furthermore, temperature also affects the solubility of salts. For example, the solubility of NaCl increases with a rise in temperature, whereas an inverse relation can be observed for $CaCO_3$, $Mg(OH)_2$, and $Ca_3(PO_4)_2$. Investigations suggest the inverse relation of solubility of alkaline salt with temperature, wherein water dissociates into hydrogen and hydroxide, forming scales; such dissociation increases at higher temperatures (Morel et al. 1993). The salts with negative relation ($CaSO_4$ and $CaCO_3$) with temperature also get saturated in the salinated feed solutions ($CaSO_4$ concentration is higher in seawater, while $CaCO_3$ concentration is higher in groundwater sources). Altogether, studies highlight a raise in scaling as the operating temperature increases (Martínez-Díez and Vazquez-Gonzalez 1999).

6.4.3 Role of Dissolved Gases in Membrane Distillation Performance

Relatively, all feeds in membrane distillation contain dissolved gases, most of which contribute to both scaling and fouling. Dissolved gases in feed streams facilitate the breakdown of bicarbonates and carry them along the vapors into the membrane pores, thus increasing the diffusive resistance of water vapors. These gases hinder the permeate flow and reduce concentration polarization and scaling (Liu et al. 1998). The dissolved gas along the feed stream occupies the membrane pores, thereby increasing the mass transfer resistance to water vapor and also adding to the mass transfer resistance in the air gaps of membranes. The effect is to reduce the condensation heat transfer rate, possibly creating the system mass transfer limited on the air side, thus reducing the overall vapor flux. Interestingly, the lack of dissolved gases can maximize the membrane wetting by removing the air trapped in the membrane pores; that has been experimentally confirmed by Schofield et al. (1990). In general, the dissolved gases present in the feed occupy the pores and create a barrier for foulants and scaling agents. Therefore, decreasing the dissolved gases by deaeration technique or other means may increase fouling propensity.

6.4.4 Superhydrophobic Membranes for Preventing Wetting

Although surface modification can reduce membrane wetting, it changes the wettability of membrane surface affecting the bulk properties. When hydrophobic membranes are modified to superhydrophobic surface, the surface roughness increases with higher contact angle; however, it has less effect in maximizing the

Table 6.6 Techniques applied in membrane fabrication for prevention of pore wetting in membrane distillation operation in order to enhance the membrane hydrophobicity which is the basic criteria of distillation membrane

Methodologies	Approach	Objective	References
Membrane casting with additional layer	Addition of hydrophilic layers	To inhibit a transport of amphipathic molecules, however, includes more resistance than a porous hydrophobic coating	Bonyadi and Chung (2007), Ray et al. (2018b), Ray et al. (2017a), Ray et al. (2017), and Tijing et al. (2014)
Membrane fabrication with simultaneous incorporation	Incorporation of perfluorinated polymers	To increase membrane hydrophobicity by reducing surface-free energy	Lalia et al. (2013), Prince et al. (2014), and Ray et al. (2016)
Membrane fabrication with blending technique	Doping of functionalized hydrophobic agents (nanoparticles)	To enhance membrane hydrophobicity by increasing the membrane contact angle and minimize the surface pore size	Dong et al. (2015) and Ray et al. (2018d)
Membrane modification	Incorporation of carbon-based micro- and nanomaterials	To enhance the membrane surface roughness maximizes the contact angle	Dumée et al. (2011), Gethard et al. (2010), and Ray et al. (2018d)
Membrane modification	Physical modification	To enhance membrane surface roughness maximizes the contact angle	Liao et al. (2013), Ray et al. (2018d), and Sinha Ray et al. (2020)

liquid entry pressure (Ray et al. 2018b). Unlike contact angle (surface property), liquid entry pressure is affected by both surface properties and the membrane pore size (Guillen-Burrieza et al. 2016; Mosadegh-Sedghi et al. 2014; Ray et al. 2018a). Table 6.6 highlights the techniques implied to tackle membrane wettability and enhance the membrane hydrophobicity or improve the self-cleaning properties.

As it has been observed that superhydrophobic membranes not only control the membrane wetting issue to a certain extent but also show higher evaporation efficiency that results in higher water flux, Figure 6.10 indicates the key roles of superhydrophobic membrane that could offer stable performance in membrane distillation operation. In short, these superhydrophobic surfaces are of special interest because of anti-sticking, self-cleaning, and anti-wetting properties.

6.4.5 Alternative Techniques

Pre-treatment of feed is one of the most efficient procedure for the reduction of fouling as it acts as the primary set of defense. Although appropriate pre-treatment escalates the permeate flux and rejection percentage, ineffective pre-treatments can have counteraction, wherein high fouling rates, reduced recovery rates, and increased

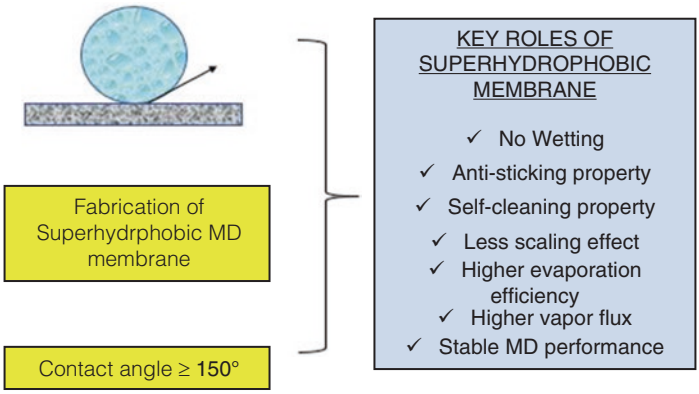

Fig. 6.10 Various roles of superhydrophobic membranes for membrane distillation operation that shows anti-wetting property, self-cleaning property, less scaling effect, and higher evaporation efficiency

operating pressure could lead to poor permeate quality and lower longevity of membrane. The efficiency of pre-treatment is correlated to parameters as agents or catalyst used, temperature (feed and treatment), concentration of impurities and suspended solids/solution and foulant properties, and the characteristics of the membrane. Overall, filtration, anti-scalants, flocculation, chlorination, and boiling (thermal water softening) are some of the most common pre-treatment methods implied both industrially and experimentally (He et al. 2009).

Pre-treatment can change the physicochemical or biological characteristics of the feed stream leading to lower fouling. For filtration to be applied, the feed must contain suspended solids or microbes, wherein reverse osmosis, nanofiltration, and ultrafiltration are the most dominant method of filtration. According to Meindersma et al. and Karakulski et al., ultrafiltration proves to be an effective method for the removal of suspended solids and colloids, whereas nanofiltration is predominantly used to reject organic materials and remove hardness from feed water (Karakulski et al. 2002; Meindersma et al. 2006). When speaking of river, the method is most commonly used to tackle the salinity of feed stream and seawater. Pre-treating seawater reduced the nutrient content of the stream implying a reduced secondary biofilm formation (Gryta 2010).

One of the other pre-treatment methods is coagulation that alters the stability of the solids and further agglomerates into heavier particles. This method proves to be very efficient to reduce pore blocking. The larger molecules that are swept over the membrane are unable to occupy the smaller pores and get deposited over the surface of the membrane. Although it might seem to be the cause of cake layer formation, it checks over pore blocking that induces fouling in membrane. Additionally, the less expensive and easily approachable nature of the method makes it more demanding. When the feed steam is groundwater, generally, thermal treatment or water softening is found to be the most convenient method. As the name suggests, this method

Table 6.7 Alternative techniques to minimize fouling of membrane that includes pre-treatment approach, optimization of operational conditions, and chemical cleaning

Approaches	Minimization methods	Outcomes	References
Pre-treatment	Coagulation Precipitation Membrane filtration Chlorination Sonication Boiling	Change in the biological as well as physicochemical properties of the feed stream and reduce the concentration of foulant	Chen et al. (2006), Fan et al. (2008), Hilal et al. (2004), and Tabatabai et al. (2014)
Optimization of operational conditions	Bubbling of gas	Increase in shear rate at the surface of the membrane and remove the fouling layer	Guillen-Burrieza et al. (2016) and Tijing et al. (2015)
	Temperature and flow reversal	Inhibit the homogeneous precipitation of salts	
Chemical cleaning	Using of acid as well as base	Rinsing with acid removes the inorganic scaling, whereas rinsing with base removes organic and biofouling	Chen et al. (2003)

primarily focuses on the removal of hardness of the feed stream. This method successfully reduces scaling caused by $CaCO_3$ and other salts to precipitate by heating. Also, vey less mentioned, this method also reduces the amount of CO_2 in the feed stream. The drawbacks of the method account for its expensive and extensive energy-consuming nature. Table 6.7 indicates the general approaches to minimize fouling in membrane distillation operation that includes pre-treatment process, optimization of operational conditions, and chemical cleaning techniques.

Over the past few decades, silica fouling became the serious issue that results in lower recovery rate during the membrane distillation operation. Typically, solubility of silica depends on both temperature and pH. However, there are some useful approaches that may control the silica fouling in distillation operation which are as follows:

- Inhibition of silica polymerization (Amjad and Yorke 1985)
- Raising solubility of silica as it forms (Meier and Dubin 1987)
- Using of polymeric dispersants to disperse precipitated silica- and silicate-based compounds (Freese 1993)

In fact, there are a number of fouling control strategies that can be applied in form of online techniques and off-line techniques. Apart from pre-treatment process, chemical cleaning, and optimization of operational conditions, there are some other options such as online techniques and off-line techniques. Basically, online strategies can be referred as utilization and optimization of appropriate additives in the feed stream, whereas off-line fouling control strategies represent the manual methodologies. So, online fouling control strategy includes the following additives: (a) anti-scalants, (b) inhibitors, (c) acids and bases, (d) dispersants, and (e) air

bumping (Ginzburg et al. 2008; Kazi 2012). The following techniques are available as far as off-line fouling control strategy is concerned: (a) manual cleaning, (b) liquid jet, (c) steam, (d) air jet, and (e) chemical and physical cleaning (Kazi 2012). This information will help other researchers to mitigate fouling propensity particularly in membrane distillation.

Over the past decade, a lot of attention was focused toward the mitigation and prevention of membrane fouling. As mentioned in the previous section, plenty of conventional methods can be used to mitigate the concern of fouling, but very recently, nonconventional methods for the pre-treatment are being used to promote the concept of reusability and minimize the energy requirement. Some of the nonconventional methods are mentioned below.

Corresponding to Gryta et al. and Yang et al., magnetic water treatment (MWT) proves very responsive for wastewater treatment from heat exchangers of power plants (Gryta 2011; Yang 2005). According to Hsu et al., the magnetic field decelerates the nucleation and enhances the crystal growth rate (Hsu et al. 2002). This modifies the crystal morphology and forms deposition layers that can be simply washed off. Another mitigation technique is to treat the membrane used and alter the surface morphology and consequently the surface properties of the membrane. In recent years, studies have highlighted the importance of making a membrane superhydrophobic. Due to superhydrophobicity of the membrane, very less nutrient retention is possible on the membrane surface. Correspondingly, diminished microbial growth was very evident according to recent studies. Generally, it can be understood from literature that hydrophobicity tackles problems related to biofouling. Most of the method mentioned above are done at both industrial and laboratory scale but are still not commercially available. Chemical cleaning is the most implied commercially available method to counter fouling. Acids, alkaline solutions, surfactants, and chelating metals are few agents used for chemical treatment. Use of acids (weak and strong) in membrane distillation is very significant to clean the scales over the membrane surface. Though this method cannot be used to tackle the problem of pore blocking as it might cause serious damage to the membrane internally, cleansing with alkaline solution could be used to reduce organic fouling. Generally, rinsing or wash-off method can only be used when the scales are deposited on the surface of the membrane. However, this method might have an adverse effect on pore blocking and could result in membrane wetting if rinsed continuously; therefore, complete recovery is quite impossible in this case. Rinsing utilizing both acid and base solutions leads to the removal of most of the deposition, recovering the hydrophobic characteristic of the membrane (Gryta 2008b).

6.5 Conclusion

In this present chapter, the versatility of membrane distillation for producing reusable water has been described thoroughly. The unique beneficial features of membrane distillation, viz., higher recovery and higher rejection of nonvolatile solutes,

prove membrane distillation to be highly efficient. Despite of the versatility of membrane distillation, fouling and wetting are of significant concern. The current scenario demanded a detailed analysis of scaling, fouling, and wetting that limit the feasibility of membrane distillation. Earlier investigations suggest lower feed temperature and turbulent flow reduce the risk of scaling, for example, $CaCO_3$ and $CaSO_4$ that are inversely soluble at increased temperature. Therefore, maintaining an optimum operating condition is an important factor in membrane distillation. Meanwhile, advanced foulant detection techniques, long-term usability, and real-time monitoring facilitate the prediction of types and intensity of fouling. Additionally, the future demands the application of anti-scalants and foulants to increase the effectiveness of membrane distillation. Furthermore, research on novel membrane materials to enhance the membrane rejection and integrity will allow a wider growth of membrane distillation technology. Nevertheless, it is important for the performance of these novel distillation membranes to be evaluated in terms of its fouling reduction capacity.

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Chapter 7
Biotechnological Applications of Immobilized Microalgae

Svetlana Vasilieva, Elena Lobakova, and Alexei Solovchenko

Contents

Abstract Nowadays, biotechnology of photosynthetic organisms sees an upward trend in usage of immobilized cells to accomplish diverse goals ranging from production of value-added molecules to destruction of unwanted contaminants. This trend exists for a good reason: from the standpoint of microalgae (MA) large-scale cultivation, immobilization provides several important advantages. Namely, the immobilized cell culture is generally more robust than suspended one, there is no need for a costly cell-harvesting procedure, and overall cultivation process becomes more manageable in case of immobilized cells. The applications of immobilization are based on the natural capacity of the microbes to adhere to the substrate and to each other. This chapter is dedicated to the biotechnological use of immobilized microalgal cells; its benefits and caveats are discussed. Special attention is paid to biological basis of the cell immobilization and the biology of immobilized cells.

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7.1 Introduction

Photosynthetic organisms including cyanobacteria and eukaryotes (commonly referred to below as microalgae) exist in their natural habitats as biofilms incorporating the cells of heterotrophic microorganisms and other microalgae embedded in a matrix of the extracellular biopolymers. The cells in the biofilms form granules, clusters, flakes, and other aggregates attached to the substrate or suspended in the aqueous media. Therefore, the immobilized state is widespread, if not ubiquitous, form of microalgae existence in nature (Coserton et al. 1995).

> Currently, algal cells attached to assorted carriers are mostly used for production of biomass and value-added metabolites, obtaining biohydrogen, and bioremoval of mineral nutrients, metals, or dangerous low-molecular pollutants from wastewater streams. The immobilized microalgae can also be incorporated into biosensors for the assessment of water pollution degree (Mallick 2002; Eroglu et al. 2015). A topical issue of mass cultivation of microalgae is harvesting of the biomass. Currently employed approaches like filtration, centrifugation, and flocculation are energy- and/or time-consuming. Immobilization of the cells streamlines considerably harvesting of the biomass.

The main strengths of immobilized cells as compared with free (suspended) cell are the improved substrate utilization, increased volumetric productivity, and enhanced resistance to unfavorable factors, e.g., extreme pH, temperatures, and toxic compounds. The major disadvantage of immobilization, compared with cultivation of suspended microalgae, is a higher cost. It should be emphasized that in real conditions, the successful application of immobilized MA is defined by optimal carries and immobilization technique (Tables 7.1 and 7.2).

In this review, we summarize the evidence on immobilization of microalgae on diverse carriers with a focus on immobilization effects on the cell metabolism and viability and advantages and shortcomings of the immobilized microalgae cell application in photobiotechnology.

7.2 Natural Biofilm as the Prototype of Artificially Immobilized Microalgae

In nature, algae-bacteria associations are stabilized mainly by microalgae controlling their formation and productivity (O'Toole et al. 2000). The microalgae play central role since they possess certain cell surface structures (mucous capsules or colonial mucus) and produce exometabolites (Zvyagintsev et al. 1993; Branda et al. 2005; O'Toole et al. 2000). The latter promote growth and viability of other

Table 7.1 Microalgae immobilization techniques

Techniques	Advantages	Limitations	References
Immobilization with a carrier			
Physical adsorption	Easy for application Repeated or continuous use Not impede cell functioning	Cell leakage	Laurinavichene et al. (2008), Abe et al. (2008), de la Noue et al. (1988), Da Costa et al. (1996), Akhtar et al. (2008), Gross et al. (2015), Schlutze et al. (2018), Cheng et al. (2014) and Zhang et al. (2017)
Covalent binding	No cell leakage	Loss of cell activity	Moreno-Garrido (2013), Codd (1987) and Seki and Suzuki (2002)
Entrapment	High cell density Resistance to toxic compounds and contamination	Less light supply Mass transfer limitations	Kosourov and Seibert, 2009, Travieso et al., 1992,; Zhang et al. 2008, Hameed and Ebrahim, 2007, Garbayo et al. 2000, Bayramoğlu et al. 2006 and Nakajima, 1982
Self-immobilization	Easy for application Low cost Easier separation	Changes in cell composition	Papazi et al. (2010), Schenk et al. (2008), Wu et al. (2012), Zhang et al. (2012), Vandamme et al. (2011), Lee et al. (2009), Salim et al. (2011), Schenk et al. (2008) and Xie et al. (2013)

members of the associations. The microbial cells incorporated in the extracellular matrix of the biofilm are less mobile similarly to artificially immobilized cells.

Microalgae are functionally linked with other members of associations. Intercellular communication of the association partners is crucial for regulation and stability of these consortia. These communities are exemplified by stromatolites and cyanobacterial mats—the oldest (3.5 billion years old) and the most evolutionarily successful form of life (Gerasimenko and Zavarzin 1993; Coserton et al., 1995).

7.2.1 Biofilm Formation Is an Efficient Strategy of Microorganism Survival

The overwhelming majority of prokaryotes normally form biofilms. Their formation is a complex, highly regulated process. Inside the biofilm, organisms are able to withstand adverse chemical, physical, and biological conditions—including dehydration, extreme temperatures, nutritional deficiency, ultraviolet irradiation, and effects of toxic substances (Romanova and Gintsburg 2011; Flemming et al. 2016). The net result is the stabilization of the microenvironment of the cells (Lewis 2005). In view of this, the formation of biofilm communities is indeed a widespread strategy for the survival of microorganisms in nature conditions.

The biofilm is based on the principle of cooperation, a basic principle of evolution (Zavarzin 2003). The members of the microbial biofilm community produce the

Table 7.2 Physiological changes in immobilized microalgae

Physiological response	Microalgal species	Immobilization techniques	References
Decreased growth	*Skeletonema costatum* and *Heterocapsa* sp., *Tetraselmis suecica*	Entrapment in alginate beads	Moreno-Garrido et al. (2005) and Pane et al. (1998)
Increased growth	*Chlorella minutissima*, *Pavlova lutheri*, *Haematococcus pluvialis* *Dunaliella bardawil* *Synechococcus sp.* *Chlorella* spp., *Anabaena* spp., *Chlorella pyrenoidosa*	Entrapment in carboxymethylcellulose gel	Joo et al. (2001)
		Entrapment in capsules of chitosan	Aguilar-May et al. (2007)
		Entrapment in alginate beads	Rai and Mallick (1992) and Huang et al. (2000)
Longer lag period; the double increase in chlorophyll content	*C. vulgaris*	Entrapment in carrageenan	Lau et al. (1998)
Increased chlorophylls, carotenoids, and lipid contents	*Botryococcus braunii* and *B. protuberans*, *Chlorella* spp.	Entrapment in alginate beads	Singh (2003) and de-Bashan et al. (2002)
Improved production of glycerol	*Dunaliella salina*	Entrapment in the agar gel	Thakur and Kumar (1998)
Increased production of marennin	*Haslea ostrearia*	Entrapment in agar gel	Lebeau et al. (1998)
Increased H_2 production yields	*Calothrix* 336/3, *Anabaena* PCC 7120, *Chlorella* sp., *Synechocystis* sp. PCC 6803	Entrapment in thin Ca^{2+}-alginate hydrogel films and beads	Kosourov and Seibert (2009); Song et al. (2011); Leino et al. (2012); Touloupakis et al. (2016)
Increased H_2 and NH_3 production yields	*Anabaena azollae* and *Mastigocladus laminosus*	Attachment to polyvinyl or polyurethane matrixes	Brouers and Hall (1986)
Decreased keto acid production	*C. vulgaris*, *Anacystis nidulans*	Entrapment in the agarose gel	Wikström et al. (1982)
Enhanced oxygen evolution	*Botryococcus braunii* and *B. protuberans*	Entrapment in calcium alginate beads	Bailliez et al. (1985); Yashverry (2003)
Increased cell membrane permeability	Chlamydomonas reinhardtii	Entrapment in barium alginate beads	Santos-Rossa et al. (1989)

(continued)

Table 7.2 (continued)

Physiological response	Microalgal species	Immobilization techniques	References
Enhanced storage capacity	*Haslea ostrearia*, *Euglena gracilis* *Amphidinium carterae*, *Chaetoceros ceratosporum*, *Emiliania huxleyi*, *Phaeodactylum tricornutum*, *Skeletonema costatum*, *Thalassiosira pseudonana*	Entrapment in alginate beads	Gaudin et al. (2006); Tamponnet et al. (1985); Hertzberg (1989)

exometabolites which other members can use as a nutrient source (Orvain et al. 2003; Hmel and Metlitskaya, 2006). Different microorganisms within the biofilm communicate by means of chemical signals. These signals control the expression of genes in the microbial cells. Therefore, the chemical signaling constitutes an important mechanism of control of the community structure, stress acclimation, and morphogenesis (Branda et al. 2005). The biopolymer matrix is normally a polyanionic hydrogel (Wingender et al. 1999) composed from proteins and polysaccharides (< 85%) with inclusion of minor amounts of lipids and nucleic acids (Branda et al. 2005). The noteworthy example of microalgae immobilization in nature is the inhabitance of transparent gel-like structures on the surface of animals like hydroids, mollusks, nematodes, and clutches of axolotl. Animals provide the microalgae with a shelter against adverse environmental conditions but at the same time transmit a sufficient amount of light for their photosynthesis. In return, the microalgae supply organic nutrients to the animal host, facilitate the mineralization of covers and its pigmentation, and augment the synthesis of the protective mucus (Trench 1993).

In view of the outline given above, the immobilization in artificial systems is expected to increase tolerance of the microalgal cells to abiotic stresses and hence to help their biotechnological application. This is a key advantage of the immobilized microalgae in comparison with suspended cultures.

7.3 The Immobilization Techniques for Microalgae

The idea of microorganism immobilization was originally developed from enzyme's attachment on different carriers, and they both have the similar approaches. However, the techniques and carriers for living cell immobilization should not be damaging (Willaert 2017).

The currently used techniques of cell immobilization are divided into two types: immobilization on a carrier surface and self-immobilization. It is essential to determine which technique is most appropriate for the specific biotechnological process. For example, cell entrapment in gels is not suitable for production of primary

metabolites, as the cell growth in polymeric gels is often limited. Self-immobilization of cells is widely used for low-cost products, such as biofuels. It should be noted that most widely used methods intended for heterotrophic microorganisms are applicable to microalgae immobilization as well if they will not restrict the light propagation in the cultivation systems.

Immobilization serves to maintain a high proportion of metabolically active cells and hence to increase the efficiency of the biotechnological processes. In many cases, immobilization of whole cells can improve conditions for cell differentiation and cell communication and also provide a favorable microenvironment, nutrient concentration gradient, so that the metabolic activities of microalgae cells can be increased. The main advantages of microalgae cell immobilization over the free cell systems are the following (Willaert 2017; Moreno-Garrido 2008; De-Bashan and Bashan 2010):

- Higher cell densities
- Easier biomass harvesting from cultural media
- Repeated or continuous use
- No mixing requirements
- Cell growing at higher dilution rates
- Higher resistance to toxic compounds
- Enhanced resistance to shear stress
- Shortening of the culture growth lag
- Improved substrate utilization
- Increased productivity per unit of the reactor volume
- Reduced risk of contamination
- Better tolerance to the inhibition by end products
- Improved genetic stability
- Improved conditions for cell differentiation and cell-to-cell communication

7.3.1 Immobilization Techniques with a Carrier

Current immobilization techniques with support materials or carriers are classified into two key types—passive and active immobilization. The passive immobilization is the process of cell attachment to a carrier that harnesses the natural ability of microorganisms to adhere to the solid surfaces (Moreno-Garrido 2008). This is the most simple and least mildly stressful method of microorganism immobilization mimicking the cell attachment in nature.

In opposition, the methods of active immobilization are the process of compulsory confinement of cells within the bulk of the carrier (López et al. 1997) and carried out mainly via entrapment of microalgae in different gels. The incorporation of microalgae in beads or sheets made of polymers can provide the increase of cell density and offer additional protection from contamination by external microorganisms and unfavorable environment (Sinitsin et al. 1994). Unicellular and small colonial algal forms are more appropriate for the active entrapment, while the adsorptive

immobilization is more preferable for filamentous forms of microalgae (Trevan and Mak 1988).

Apart from the immobilization techniques, the choice of carrier materials for microalgae attachment is another crucial decision. Nowadays, there is a wide choice of natural and synthetic materials for microalgae immobilization as it is a rapidly developing area of biotechnology. The preeminence of natural carriers is biocompatibility, hydrophilicity, and ease of recycling. Their disadvantages are high cost and lower stability in wastewater as compared to synthetic carriers. As a rule, natural polymers are less resistant to biodegradation. However, the diffusivity in natural materials is higher than in synthetic ones, and they are safe for the environment (Leenen et al. 1996). Several natural polymers such as loofa, sphagnum, turf, glass, wood, and natural polysaccharides (alginate, cellulose, carrageenan, chitosan) could be used for passive immobilization techniques. Synthetic polymers such as polyacrylamide, polyurethane, polyvinyl chloride, polypropylene, polysulfone, and epoxy resin have been experimentally used for microalgae attachment (Moreno-Garrido 2008). Anyway, the carrier material must be hydrophilic, not impeding the mass transfer in the cultivation system or in wastewaters.

The ideal carrier for microalgae should meet the following criteria (Willaert 2017; Moreno-Garrido 2008; Hameed and Ebrahim 2007; Mallick 2002; Eroglu et al. 2015):

- Low cost
- Possess a high affinity to the cells
- Not affect the cell functioning
- Photo transparent
- Hydrophilic
- Nontoxic.
- Possess high retention capacity during prolonged cultivation
- Resistant to disruption by cell growth
- Conductive for a sufficient mass transfer
- Possess mechanical, chemical, and thermal stability during operation
- Easy for application in the immobilization procedure
- Low affinity to contaminations
- Biodegradable or capable of recycling
- Safe for the environment
- Possess a large surface area for cell attachment
- Can be prepared in specific particle size and shape

7.3.2 *Passive Immobilization*

Many species of microalgae have a natural ability to attach to solid surfaces and propagate on them (Robinson et al. 1986). The optimal carrier for passive attachment of microalgae should have a high affinity toward the surface structures of microorganisms and cause minimal damage.

Reportedly, the passive attachment of cells to inorganic or organic carrier materials is achieved via chemical (covalent binding) or physical (ionic, van der Waals, electrostatic, hydrophobic) interactions (Sinitsin et al. 1994). It is expected that the adhesion behavior of viable cells is affected by chemical and physical properties of the carrier matrix (Characklis et al. 1990). An increased attachment of microorganisms to rough materials as compared to smooth ones was revealed and explained by physical phenomena, e.g., the increased convection for rough surface in aquatic media. The cracks and holes on the surface create zones with slower liquid velocity, so the algal cells can settle on and attach to the surface (Cao et al. 2009). Rough texture also decreases the shear forces directed on the immobilized cells (Cui et al. 2014). Moreover, the rough surfaces with holes, cracks, and protuberances provide more area for microalgae immobilization when compared to smooth polymeric materials. Particularly, the water-carrier material contact angle (Irving and Allen 2011) and surface energy (Christenson and Sims 2012; Genin et al. 2014) have been widely researched for their influence on the process of cell attachment.

Microalgae cell attachment mechanism is thought to be different in different groups of microalgae (Sekar et al. 2004). The cell attachment is more efficient in microorganisms possessing different surface structures such as flagella, pili, epistructures (Dufrêne 2015; Vadillo-Rodríguez et al. 2004). In this case, the repulsive force between the cells and a carrier decreases due to reduction of the initial contact area (Sinitsin et al. 1994). Tosteson and Corpe (1975) reported that the attachment ability of algal cells depends on their capacity to excrete adhesive substances such as exopolysaccharides. Woods and Fletcher (1991) studying the attachment of four marine diatoms found that the variation in rates and strength of adhesion was due to the cell's capability of rapid synthesis and excretion of exopolysaccharides. Most of the diatoms attach to the solid surface by the production of extracellular polymeric substances in the form of stalks, apical pads, mucilage pads, and cell coatings (Hoagland et al. 1993). It is shown that the lack of nutrients in media triggered the secretion of extracellular polymer by microorganisms resulting in the increasing of their immobilization efficiency (Orvain et al. 2003; Zhang et al. 2014). It was revealed in the recent study (Kreis et al. 2018) that the unspecific adhesion of *Chlamydomonas reinhardtii* to surfaces can be reversibly triggered by switched-on and switched-off illumination in the cultivation system.

Effects of environmental and/or physiological conditions on the attachment mechanisms of different microalgae have been intensively studied (Sekar et al. 2004). Reportedly, the attachment of *Navicula amphibia* was directly proportional to the culture density. The growth stage of microalgae and cultural media composition are also shown to be important for the cell attachment ability. Zaidi and Tosteson

(1972) observed different adhesion rates of *Chlorella* cells during different growth stages and found that the cells in logarithmic (exponential) phase attached more vigorously. The cells of *Anabaena doliolum* exerted the maximal capability of immobilization at the exponential phase of the culture growth; this capability declined or was retained at the stationary phase (Mallick and Rai 1994). By contrast, the efficiency of *C. vulgaris* cell immobilization on polyethylenimine-based materials was higher in stationary-phase cultures; in the exponentially growing cultures, the attachment efficiency was 23–27% lower (Vasilieva et al. 2018). It is likely that the increased polysaccharide layer that *C. vulgaris* cells develop at the stationary phase facilitates electrostatic interaction between the cells and the polycationic sorbents. Interestingly, live cells attached the carriers more effectively when compared to heat-killed cells, showing the implementation of active mechanisms in the process of algae immobilization (Sekar et al. 2004).

A major problem common to immobilization of viable cells is their leakage from the surface of the immobilization matrixes. For irreversible steady attachment of microalgal cells to carrier surface, the latter can be pretreated. The use of carriers impregnated with ligands containing amine groups for immobilization of microorganisms on glass surface (Tsygankov et al. 1994) results in 800 times increase in the number of attached cells as compared to untreated glass.

Polymers based on cross-linked polyethylenimine were applied for *C. vulgaris* cell immobilization. A considerable rise of the immobilization efficiency was documented via electrostatic interaction of the negatively charged microalgae surface and positively charged amino groups of sorbents (Vasilieva et al. 2018). Natural materials such as chitin or chitosan are also widely used for steady binding of microalgae cells. It was also revealed that the effectiveness of microalgae and polycationic polymer interaction depends on pH and ionic strength of the surrounding media (Codd 1987).

The covalent binding with toxic reagents (dialdehydes, diisocyanates) is more suitable for immobilization of dead microalgae cells (Moreno-Garrido 2008) as the strong chemical interaction damages the cell surface and reduces viability of cells.

As compared to nonporous materials, porous carriers are also more preferable for adsorptive immobilization as they have an open network of cavities through which nutrient medium may pass (Willaert 2017). The porous materials can be developed from fiberglass mats, cotton fiber, polyester fibers, and reticulate polyurethane foam. Among all, polyurethane foam has advantages as a matrix such as being highly porous and nontoxic to the microalgae cells. Immobilization of *C. vulgaris* cells was achieved by their migration into polyurethane foam blocks (Travieso et al. 1996). Loofa biomass is a commonly used natural material (Akhtar et al. 2004) consisting of a fibrous network with a high degree of porosity. It possesses a high specific surface area and mechanical strength combined with biodegradability, nontoxicity, and low cost.

When microalgal cells grow attached to porous materials, they still can be confined inside the large pores. In this case, the system with immobilized microalgae can be compared to gel entrapment of microalgae which is the widespread immobilization technique (Willaert 2017).

Immobilization in porous materials has the following main advantages over the gel-entrapped cells: (i) the immobilizing particles better resist compression, which unavoidably takes place in bioreactors; (ii) the immobilization occurs under mild conditions, and for this reason, microalgae cells retain their viability; and (iii) it is simpler and cheaper, so it better suits for large-scale immobilization. Drawbacks of the immobilization on porous carriers are the following: (i) microalgae cannot be completely immobilized due to cell leakage from the carrier surface, and (ii) if the pores are large and deep, the internal mass transfer limitation becomes significant at high local cell densities leading to decreasing of cell metabolic activity.

Thin-layer immobilization offers a short light travel distance through the attached microalgae. It is a significant advantage for algal biotechnologies because such systems support much higher cell densities because short light path increases light transmission and hence the potential culture density (Solovchenko et al. 2016). The short light path, high surface-to-volume ratio, efficient mass transfer, and high culture density in the late exponential phase are typical for thin-layer systems (Doucha and Lívanský 2014). Essentially, the immobilized high-density microalgal culture represents a close approximation to a high-productivity thin-layer photobioreactor combining the advantages of this cultivation system and immobilized algae cultivation.

Various natural and synthetic carriers are used for adsorptive immobilization of microalgae in such systems (Gross et al. 2015). The attachment materials have to resist moist conditions, withstand the physical force applied during mechanical harvest, and allow active propagation of microalgae cells leading to the biofilm development. Recently, printing paper (Schultze et al. 2015), cellulose acetate membrane (Liu et al. 2013), cotton duct (Gross et al. 2015), and lignocellulose materials such as pine sawdust, rice husk, sugar bagasse, and oak sawdust (Zhang et al. 2017) were used for microalgae biofilm formation in thin-layer cultivation system.

7.3.3 Active Immobilization

The method of cell entrapment in the bulk volume of a carrier is the most widely used technique for microalgae immobilization (Mallick 2006). In this method, cell suspension is mixed with natural or synthetic monomers, and the mixture is solidified to produce a polymeric gel of various forms. Polymerization can be achieved by physical-chemical treatments such as cross-linking of the monomers with divalent cations (Cohen 2001) or polyvinyl alcohol and lactides (Moreno-Garrido 2008), photodimerization, iterative freezing and thawing, and thermal gelation (Willaert 2017). Entrapped microalgae are used mainly for biotechnological applications in biosynthesis of secondary metabolites and bioremediation of waste, as test organisms in ecotoxicology, and for removing biogenic elements, e.g., phosphorus and nitrogen, and heavy metals from wastewater (Eroglu et al. 2015).

Synthetic polymers (acrylamide resins, polyurethanes, polyvinyl alcohol, polypropylene), proteins (gelatin, collagen), or natural polysaccharides (chitosan,

agarose, agaropectin, cellulose, carrageenans or alginates, polygalacturonic acid) are suitable for this purpose (Moreno-Garrido 2008). Substrates and products can diffuse in and out of the gel beads, while cells are retained inside the beads. Nevertheless, the mass transfer kinetics is reduced in most entrapment systems (Aksu et al. 2002) as compared to that in suspended cells. It should be noted that natural polymers have higher nutrient/product diffusion rates as compared to the synthetic polymers (Leenen et al. 1996).

Agar is one of the most widespread materials for polymeric bead formation (Burdin and Bird 1994; Moreno-Garrido et al. 2005). Beads from calcium alginate also found extensive use for microalgae immobilization (Eroglu et al. 2015). These beads do not reduce growth of the entrapped microalgae by limiting the light propagation, and they are not toxic to microalgal cells (Leenen et al. 1996). Despite these advantages, alginate beads do not uphold their polymeric structure in the wastewaters with high nutrient content and the presence of K^+ or Mg^{2+} (Mallick 2006).

It was revealed that the mechanical resistance of natural polymers can be strengthened after mixing them with chitosan (Zheng et al. 2005), Japanese konjac flour (Kaya and Picard 1996), polyacrylamide (Kuu and Polack 1983), polyethyleneimine, glutaraldehyde, silica, genipin, and polyvinyl alcohol (Willaert 2017) resulting in a more mechanically stable materials for microorganism entrapment.

Apart from mass transfer limitations, the negative effect of microalgae immobilization in polymeric beads includes the decreasing of the volume-to-surface ratios and light supply to entrapped cells. To overcome these problems, the microalgae entrapment in thin polymeric films is recently proposed to provide sufficient light and nutrients to phototrophic microorganisms for increasing light-to-product conversion efficiency. Kosourov and Seibert (2009) immobilized *C. reinhardtii* inside alginate films for the photoproduction of hydrogen; a few species of cyanobacteria were entrapped in the hydrated latex coatings (Bernal et al. 2014). *Chlorella* sp. entrapped in calcium alginate sheets was used to remove inorganic nutrient compounds from domestic wastewater.

Various nanofabrication processes have been applied for creation of new carriers on the basis of electrospun nanofibers (Eroglu et al. 2015), laminar nanomaterials such as graphene, and graphene oxide nanosheets (Wahid et al. 2013) for the thin-layer immobilization of microalgal cells. The new nanofiber materials possess high level of porosity and surface-to-volume ratio, and their synthesis techniques allow to create polymers of different shapes, from fibers to flat thin membranes (Crandall 1996).

Reportedly, gels and films from cellulose nanofibrils which are renewable, biocompatible, and biodegradable material offered a range of advantages for the microalgae immobilization (Klemm et al. 2011). Recently, TEMPO-oxidized cellulose nanofibrils cross-linked with Ca^{2+} or polyvinyl alcohol were applied for *Chlamydomonas reinhardtii* and *Anabaena* sp. entrapment in polymeric sheets (Jämsä et al. 2018).

7.3.4 Self-Immobilization

For the cell adsorption or entrapment with support materials, the additional costs for carriers and immobilization procedures are involved, which often makes this technology economically unprofitable. By contrast, self-immobilization process (flocculation) presents a lot of advantages over immobilization such as the following:

- More cost-effective immobilization process
- Better cell growth due to less mass transfer limitation
- Less physical restriction exerted on cells
- Easier for application in the immobilization procedure
- Higher cell densities as no support materials occupy the working volume of the bioreactors

The main disadvantage of some self-immobilization technique is that they may cause unwanted changes in cell composition of microalgae during prolonged cultivation (Benemann and Oswald 1996), so they are more appropriate for the production of primary metabolites. The use of self-immobilization for biomass harvesting can also be an efficient alternative to the time-consuming and expensive techniques such as filtration and centrifugation.

Microalgal cells can stick together to form cell aggregates naturally or under certain artificially induced conditions. In biotechnological processes, flocculation can be carried out in various methods. Chemical flocculation using salts of Zn^{2+}, Al^{3+}, and Fe^{3+} is the most used technique (McGarry 1970). Flocculation can also be induced by changing the microalgae cultivation conditions such as increasing the medium pH, nutrient depletion, and temperature changes. Increasing the medium pH value resulted in the high flocculation efficiency of up to 90% for freshwater (*Chlorella vulgaris*, *Scenedesmus* sp., *Chlorococcum* sp.) and marine microalgae (*Nannochloropsis oculata*, *Phaeodactylum tricornutum*) (Wu et al. 2012). It has been revealed that the cell aggregations did not occur in waters with calcium and magnesium deficiency (Yahi et al. 1994); thus, it was assumed that flocculation of microalgae at high pH is caused by chemical precipitation of calcium and/or magnesium salts.

Polyelectrolytes can be used to enhance the aggregation of microalgal cells. The surface of algal cells contains various functional groups (de la Noue and de Pauw 1988). They can be deprotonated or protonated depending on the pH and therefore can accumulate the surface charge. Electrical methods based on electrophoresis are also used for microalgae self-immobilization (Vandamme et al. 2012); the negatively charged surface of microalgae allows to concentrate cells by being moved in an electrical field (Zheng et al. 2012).

Bio-flocculation techniques allow the harvesting of microalgae without addition of chemical flocculants and the reuse of the cultivation medium. The flocculation of microalgae induced by bacteria (alga-bacteria flocculation) has been implemented successfully in wastewater treatment (Lee et al. 2013). The usage of poly-γ-glutamic acids from *Bacillus subtilis* for harvesting *Nannochloropsis oculata*, *Phaeodactylum*

tricornutum, *C. vulgaris*, and *Botryococcus braunii* resulted in 90% of flocculation efficiency.

However, co-cultivation of microalgae with bacteria used in this kind of flocculation approaches results in contamination of the biomass precluding its applications for food (Vandamme et al. 2012). However, the added microorganisms may contribute to the increase in lipid yields and fatty acid contents in microalgae cells, and this is beneficial for biofuel application of the biomass (Salim et al. 2011; Chen et al. 2011).

It was demonstrated that algal-fungal flocculation is the efficient way of planktonic algal cell harvesting (Muradov et al. 2015), but the subsequent separation of the fungi and algal cells challenges its scale-up process. Algal-algal-based flocculation does not demand the separation process of the harvested biomass, and energy consumption is negligible (Alam et al. 2014). Recently, the naturally flocculating diatom *Skeletonema* was used to form flocs of *Nannochloropsis* (Schenk et al. 2008). Autoflocculating microalgae, e.g., *Ankistrodesmus falcatus*, *Scenedesmus obliquus*, or the marine species *Tetraselmis suecica*, *can be used for harvesting* non-flocculating microalga. *In* comparison with other applied bio-flocculation techniques, *the* algal-algal self-immobilization does not require different cultivation conditions and prevents unwanted contaminations (Salim et al. 2011).

7.4 Physiology of the Immobilized Microalgal Cells

Over the last decade, numerous studies have identified regulatory pathways and genetic responses of the heterotrophic bacteria and yeast immobilized at carrier surfaces and within biofilms (Kuchma and O'Toole 2000), but corresponding reports for microalgae are obviously lacking in the literature. Most of the published reports are focused on the physiological responses which are considered in this section.

The cells in naturally occurring immobilized systems such as biofilms (Coserton et al., 1995) display a variety of physiological changes as compared to free cells of the same species. Part of these modifications starts during the first stages of immobilization following the cell-support contact, where microorganisms develop surface-sensing responses (O'Toole et al. 2000). The physiology of living algal cells upon immobilization changes because the attached or entrapped microalgae cells are subjected to a different microenvironment compared with the free-living cells (Junter et al. 2002).

Growth rate is probably the most studied parameter of immobilized microalgae indicative of the metabolic processes within these systems. Contradictory results have been published, showing decreased (Moreno-Garrido et al. 2005; Pane et al. 1998; Robinson et al. 1986; Hameed and Ebrahim 2007) or enhanced growth rates (Aguilar-May et al. 2007; Joo et al. 2001; Rai and Mallick 1992; Huang et al. 2000) of immobilized microalgae as compared to the free cells. The most evident hypothesis to explain a decrease in the growth rate of the immobilized cells is mass transfer

limitation. Generally, cell division rate was higher in the case of surface immobilization than for entrapped cells.

Adsorptive microalgae immobilization results in maximum interface area between the attached cell surface and the liquid medium resulting in the lowest mass transfer limitation. In case of cell entrapment, molecular diffusion of compounds through an immobilized cell matrix can be facilitated by the use of microstructured immobilization matrix as well as by capillary or active transport (Eroglu et al. 2015).

The process of polymer-based entrapment can impose harsh stress on microalgae reducing immobilized cell population, although this effect is species-specific (De-Bashan and Bashan 2010; De-Bashan et al. 2005). Thus, the cells of *Skeletonema costatum* and *Heterocapsa* sp. did not propagate in alginate beads, while the growth rate of other microalgae entrapped in alginate was similar to suspended cells (Moreno-Garrido et al. 2005). Other modifications in the immobilized cell microenvironment than nutrient/oxygen depletion include accumulation of toxic waste products and buildup of osmotic pressure (Inanç et al. 1996) that might explain the reduced growth rate for immobilized microalgae as well.

In certain cases, immobilized microalgae showed faster cell division rate than suspended cultures. This was the case when *Dunaliella bardawil*, *Pavlova lutheri, Chlorella minutissima,* and *Haematococcus pluvialis* were entrapped in 2% carboxymethylcellulose gel (Joo et al. 2001). The growth-promoting action of immobilization has been explained by protective effects of the support materials during cultivation of microalgae in unfavorable conditions. The diffusion resistance in case of gel-entrapped cells may be also beneficial by decreasing of the local concentration of inhibiting substrates. On the contrary, immobilization may provide a better bioavailability of nutrients in oligotrophic conditions by their concentration near the carrier.

It should be noted that the culture growth is often estimated via chlorophyll accumulation rate, which is able to increase in attached cells as compared to suspended ones, possibly as an adaption to self-shading. Therefore, chlorophyll measurements would probably overestimate cell number and thus lead to a certain error in the estimation of immobilized cell productivity.

Alterations in size and shape of attached or entrapped microalgae are also extensively recorded in the literature (Cassidy et al. 1996; Bailliez et al. 1986) as well as changes in size of trichomes and microalgal colonies (Mallick 2002). Immobilized colonies of *Botryococcus* were found to be 2.5 times larger than those of free-living cell colonies (Chevalier and de la Noue 1985), while *the cells of Chlorella* entrapped in calcium alginate tend to form small colonies (8–30 cells) (Trevan and Mak 1988).

Immobilization increases tolerance of microalgae to changes in temperature, pH, or ionic strength of the cell surroundings (Sinitsin et al. 1994). For example, the chitosan-immobilized cells of *Synechococcus* sp. have higher resistance to NaOH as compared to the suspended cells (Aguilar-May et al. 2007). Immobilized cultures better withstand different toxicants. Thus, toxicity of chromium and nickel ions to the cells of a nitrogen-fixing cyanobacterium *Aulosira fertilissima* was reduced considerably upon their entrapment in alginate beads (Banerjee et al. 2004). This effect

is of particular significance for environmental applications, e.g., for biosequestration and bioconcentration of heavy metals from wastewater.

Accumulation of pigments and changes in fatty acid profile are common effects for attached or entrapped cells as compared with free (suspended) microalgae (Lau et al. 1998). Chlorophyll content of *Chlorella vulgaris* cells immobilized in carrageenan gel was twice higher than in suspended culture (Lau et al. 1998). Cells of *Botryococcus braunii* and *B. protuberans* immobilized in alginate beads contain more chlorophylls, carotenoids, and lipids during the stationary growth phase in comparison with free cells (Singh 2003). Co-immobilized bacteria *Azospirillum brasilense* and *Chlorella* spp. promoted pigment and lipid accumulation (de-Bashan et al. 2002).

Higher specific production rates of immobilized microalgal cells as compared to suspended ones have been investigated for the production of secondary metabolites. Immobilization of *Dunaliella salina* in agar-agar resulted in significant enhancement of glycerol production in comparison with free cells (Thakur and Kumar 1999). Thus, agar entrapment of the marine diatom *Haslea ostrearia* augmented the production of marennin, a blue-green pigment demanded by commercial culturing of oysters (Lebeau et al. 1998). Ammonia production by *Mastigocladus laminosus* was significantly increased when the cells were confined in polyvinyl sheets (Brouers and Hall 1986).

The improvement of photosynthetic productivity was proved for several species of cyanobacteria immobilized in the hydrated latex coatings (Bernal et al. 2014). H_2-producing green algae (Kosourov and Seibert 2009; Song et al. 2011) and heterocystous cyanobacteria (Leino et al. 2012; Touloupakis et al. 2016) entrapped within thin calcium alginate sheets and beads demonstrated the increased H_2-production yields compared to suspended microalgae.

It was revealed that in the free cells, hydrogen production was largely achieved by the nitrogenase enzyme system, but in immobilized cells, it was essentially hydrogenase mediated, particularly when calcium alginate was used for entrapment of cells (Touloupakis et al. 2016). It was found that this shift was not because of nitrogenase inhibition by immobilization since the enzyme activity and stability were both increased. Immobilization of *Anabaena azollae* and *Mastigocladus laminosus* in polyvinyl or polyurethane carriers resulted in enhancement and/or stabilization of H_2 photoproduction rate (Brouers and Hall 1986). Hydrogen production by immobilized *Anabaena* sp. was increased threefold likely due to decreased cell growth and better protection against shear stress (Kayano et al. 1981).

It was supposed that the impaired mass transfer, e.g., limited nutrient availability, augments the yield of target substances, e.g., secondary metabolites such as reserve lipids or secondary carotenoids. The net result of it is channeling of photofixed carbon, ATP, and NADPH to the production of the desired end products instead of cell multiplication (Lukavský 1988).

Nevertheless, there are many examples where immobilized cultures demonstrate unchanged or even lower rate of metabolite accumulation as compared to suspended cultures. For example, polysaccharide production by *Porphyridium* is reduced by

65% (Thepenier et al. 1985), and keto acid production by *Anacystis* and *Chlorella* is reduced by 70–90% (Wikström et al. 1982).

In most cases, the change in the photosynthetic activity of immobilized phototropic microorganisms is defined by alterations of their illumination as compared with free-living cells (Lebeau and Robert 2006; Jeanfils and Collard 1983; Robinson et al. 1986). In *Chlorella*, no difference in oxygen evolution between free and immobilized cells was observed (Robinson et al. 1986). In the other studies, the immobilized cells featured a faster oxygen evolution suggesting a dramatic change of the cell metabolic activity (Bailliez et al. 1985).

There are different hypotheses explaining the observed alteration of photosynthetic activity in immobilized microalgal cells. Immobilization stabilizes protein-chlorophyll complexes in *Botryococcus* and *Euglena*: 90% of chlorophyll was retained by the immobilized cells after 3 months. Photosynthesis can be enhanced due to possible increase in the certain ion concentrations in the microenvironment of immobilized cells (Bailliez et al. 1986). The photosynthetic oxygen evolution and specific growth rate of suspended and immobilized *Scenedesmus dimorphus* cells were compared by Wang et al. (2015). The full illumination in the open pond happened only for 3-day cultivation, and biomass was very dilute (<20 g m^{-3}), whereas in the attached cultivation system, the full illumination of microalgae lasted to 10 days, and biomass density reached up to 100 g m^{-3}. The higher percentage of photosynthetically active cells might explain the cases of higher biomass productivity of immobilized microalgae as compared to the free cells.

On one hand, photosynthesis rate decreases if the immobilized cells starve of light energy due to self-shading or blocking of light by the carrier. On the other hand, the cells attached to the surface of a carrier or confined in the polymeric gels can be protected from photodamage if the illumination is in excess. The light supply to the cultures can be facilitated by usage of optical fibers incorporated into the polymeric matrix (Matsunaga et al. 1996). Long-term stabilization of the photosynthetic activities measured as chlorophyll fluorescence in chlorophyte (*Scenedesmus* and *Chlorella*) cells has also been reported. Chen (2001) showed that cells of *Scenedesmus quadricauda* retain their viability and photosynthetic activity even after 3 years of storage.

Co-immobilization of microalgae with heterotrophic bacteria also helps to lift the limitation of photosynthesis in the entrapped microalgal cells by insufficient CO_2 supply. The co-immobilized bacteria efficiently provide microalgae with the CO_2 originating from their respiration (Moreno-Garrido 2008). Microalgae-bacteria associations have already been shown to benefit from each other, with microalgae cells producing oxygen and some organic compounds that are consumed by heterotrophic bacteria. Bacteria can also excrete vitamins and hormones that can enhance the algal growth (De-Bashan et al. 2005; Gonzalez and Bashan 2000). Mouget et al. (1995) also proved that *Pseudomonas diminuta* and *Pseudomonas vesicularis* bacterial cells isolated from the cultures of *Scenedesmus bicellularis* and *Chlorella* sp. stimulated the growth of the corresponding microalgae.

As it was shown for bacteria, the process of immobilization is able to modify the membrane composition and permeability by inducing a shift of the membrane lipid

fatty acid composition, unsaturation, and hence the membrane fluidity (Jirků 1999). Unfavorable environmental conditions in immobilized systems, i.e., high osmotic pressure and nutrient limitations and/or mechanical stress, have been put forward as a putative explanation of these modifications (Junter et al. 2002). An increase in cell membrane permeability has also been observed for microalgae *Chlamydomonas reinhardtii* entrapped in alginate beads, favoring the uptake of nitrite ions (Santos-Rosa et al. 1989).

7.5 Immobilized Microalgae in Biotechnology

The mainstream applications of immobilized algal cells are biomass and value-added metabolite production, obtaining biohydrogen, and for biocapture of nutrients and metals from or destruction of the organic pollutants in wastewaters. The immobilized microalgae are also used as biosensors to assess the degree of water pollution (Mallick 2002; Mulbry et al. 2005; Eroglu et al. 2015). The problem of algae biomass harvesting is one of the main challenges in photobiotechnology. Currently employed approaches (filtration, centrifugation, flocculation) are energy- and/or time-consuming. The using of immobilized cells streamlines considerably the process of biomass harvesting. Other areas of immobilized microalgal cell application are exemplified in the following sections.

7.5.1 Biomass and Value-Added Metabolite Production

It was proved that immobilized cells of *Porphyridium cruentum* were more efficient producers of sulfated polysaccharides as compared to free cells (Gudin and Thepenier 1986). The other prominent example is the usage of *cyanobacterium Aphanocapsa halophytia MN-11* immobilized in calcium alginate gel and coated on light-diffusing optical fibers for sulfated polysaccharide production (Matsunaga et al. 1996). Cyanobacterium *Spirulina platensis* immobilized in alginate converted morphine to the alkaloid codeine (Rao and Hall 1984).

The NH_3 production by nitrogen-fixing cyanobacteria *Anabaena azollae* immobilized in polyurethane foam was enhanced during cultivation in a photobioreactor (Kannaiyan et al. 1994). The hydrocarbon production by alginate-immobilized *Botryococcus braunii* and *Botryococcus protuberans* was significantly increased during stationary growth phase. Santos-Rosa et al. (1989) revealed that *Chlamydomonas reinhardtii* cells immobilized in alginate beads sustained more stable photoproduction of ammonia during their prolonged cultivation in a photobioreactor than free-living cells. Glycerol production in alginate-entrapped cells of *C. reinhardtii* was doubled as compared to suspended cells.

Sustainable sources of energy such as biohydrogen produced by microalgae draw increasing attention during the last decades. Certain microalgae species are

able to produce hydrogen under stressful conditions, e.g., in the absence of sulfur in the cultivating medium. Sulfur starvation blocks the synthesis of photosystem II-specific proteins, inhibits photosynthesis, and eventually facilitates the hydrogen production by phototrophic cells (Melis et al. 2000). *Chlamydomonas reinhardtii* seems to be one of the most promising organisms for biohydrogen production on an industrial scale (Kosourov and Seibert 2009; Laurinavichene et al. 2008).

Cyanobacterial strains capable of nitrogen fixing are promising for evolving H_2 as a by-product of their nitrogenase activity. Significantly, nitrogenase is protected from the inhibitory effect of oxygen in heterocysts (Kosourov and Seibert 2009). It was shown (Kayano et al. 1981) that the hydrogen productivity of cyanobacterium *Anabaena* N-7363 immobilized in 2% carrageenan gel was 2.4 times higher in comparison with the suspended algal cells (up to 3.24 mmol per hour per 1 g dry gel).

Alginate bead entrapment of *C. reinhardtii* resulted in a better retention of hydrogenase activity since the alginate layer delays oxygen penetration into microalgae cells. As a result, the cells entrapped in alginate produce more hydrogen as compared to free cells (Kosourov and Seibert 2009). The rates of hydrogen production by *C. reinhardtii* cells immobilized on glass fibers and suspended cells were similar, but the period of hydrogen evolution was longer for the attached cells (Laurinavichene et al. 2008).

7.5.2 Bioremoval of Nutrients from Wastewater

Biotreatment with microalgal cultures is a promising biotechnological application for remediation of wastewater (including agricultural wastewater) as compared to currently used technologies (Solovchenko et al. 2013). Microalgae application for treatment of nutrient-rich wastewater allows to remove nitrogen and phosphorus and obtain biomass enriched with these nutrients (Mallick 2002). Fertilizer production is among the most promising methods of nutrient-enriched microalgal biomass utilization. The promising approach is usage of algal turf scrubbers for farm wastewater treatment which support the load rate of 2700 kg of N and 400 kg of P per 1 ha per year, yielding 27,000 kg ha^{-1} of dry microalgae biomass (Pizarro et al. 2006).

The approaches to nutrient capture by immobilized microalgal cells include (i) their entrapment in natural polymer gels (de-Bashan et al. 2015) and (ii) passive immobilization on the surface of biodegradable and nontoxic carriers (Abe et al. 2008). *Chlorella vulgaris* immobilized in alginate removed 80% of ammonium and 99% of phosphate from wastewater during 24 hours (Travieso et al. 1992). The cells of *Scenedesmus* immobilized in alginate sheets also effectively removed nutrients from tertiary wastewater (Zhang et al. 2008). The chitosan-immobilized cells of *Phormidium* sp. remove up to 95% of nitrates and 87% of phosphate within 24 hours (de la Noue and de Pauw 1988).

At low nutrient concentrations, their consumption by gel-entrapped microalgal cells can be limited, possibly due to decreasing the nutrient diffusion through the

immobilizing matrix. It was found (Garbayo et al. 2000) that *C. reinhardtii* immobilized in Ca alginate did not consume nitrate at a concentration below 0.14 mM, but freely suspended cells consumed it nearly completely.

Despite the limited division rate of microalgal cells in beads, the immobilization can enhance the metabolic activity of cells providing higher rate and efficiency of wastewater treatment as compared to suspended cells. Within 3 days, immobilized *C. vulgaris* cells consumed more than 95% of ammonium and 99% of phosphate, while the removal efficiency of free cells was two times lower (Hameed and Ebrahim 2007). Zhang et al. (2008) established that the cell density of immobilized microalgae was the key factor that determined the efficiency of nutrient removal. Higher rates of nutrient removal can be achieved through higher cell density in alginate gels when thickness of polymeric sheets was 2–3 mm.

Thermophile strains are particularly suitable for nutrient bioremoval at high temperatures (> 30 °C). Thermophilic cyanobacterium *Phormidium laminosum*, immobilized on hollow cellulose fibers, was applied for nutrient removal in a tubular photobioreactor at 43 °C (Sawayama et al. 1998).

The rate of nutrient uptake is enhanced if the carrier used for microalgae immobilization also binds the nutrients. For example, carrageenan adsorbs ammonium cations, while chitosan has high affinity to anions (phosphate, nitrate, and nitrite) (Mallick and Rai 1994).

Reportedly, MA can accumulate P in excess of growth requirements which is known as luxury uptake (Solovchenko et al. 2016). Luxury P uptake probably developed as an adaptive mechanism of microalgae in response to unstable P availability (Watanabe et al. 1988). The immobilization of green microalgae *Scenedesmus* sp. and *Chlorella* sp. capable of luxury P uptake (Azad and Borchardt 1970) resulted in high levels (up to 90%) of P removal from wastewater (Shi et al. 2007; Wei et al. 2008).

The nutrient removal efficiency could be significantly increased by preliminary cultivation of microalgae in nutrient-depleted media (Solovchenko et al. 2016; Urrutia et al. 1995). The study (Zhang et al. 2007) demonstrated that immobilization of *Chlorella* sp. entrapped in alginate screens for starvation-wastewater treatment process would be a prospective method for the wastewater treatment. During four cycles of starvation treatment, the nutrient removal efficiency was up to 100% because of high cell growth, but after the fifth cycle, the N and P removal efficiency decreased because of light shading and longtime starvation of microalgae.

Apart from single-species microalgae immobilization, the idea of more than one microorganism attachment on the support materials can contribute to the higher effectiveness of bioremediation process. As discussed above, algal-bacterial consortia can benefit from co-immobilization in different carriers (De-Bashan et al. 2004), as photosynthesis of the entrapped microalgae cells can be limited due to decreasing of CO_2 supply. In many cases, wastewater contains organic P species which are scarcely uptook by microalgae. At the same time, bacteria often possess enzymes to mineralize the organic P compounds and provide the higher bioavailability of P in wastewater (Lim et al. 2007).

The algae-algae immobilization is also promising. Thus, monocultures of *Rhodobacter sphaeroides* or *Chlorella sorokiniana* are unable to simultaneously remove acetate, propionate, ammonia, nitrate, and phosphate, while the mixed culture is capable of this (Ogbonna et al. 2000). It should be noted that interactions among organisms in mixed-culture photobiofilms also are not well understood (Kesaano and Sims 2014).

7.5.3 Removal of Heavy Metals

Heavy metal pollution is an increasingly important problem nowadays. Cells of microalgae can accumulate diverse elements including heavy metals in high concentrations, so they are widely used for their removal from wastewater (Nascimento and Xing 2006; Saeed and Iqbal 2006).

Adsorption of metal ions on the surface of microalgal cells proceeds via binding to cell wall and/or cytoplasmic membrane and surface structures. Efficiency of heavy metal removal depends on the microalgal cell total surface area. Accordingly, increasing the cell density by immobilization results in a significant increase of heavy metal adsorption from wastewater (Malik 2004). For removing of heavy metals, microalgae are frequently attached to the surface of various natural (alginates, chitosan, carrageenan) and synthetic (polypropylene, polysulfone, polyacrylamide) carriers. The seaweeds *Sargassum* sp. and *Ulva* sp. were also used for immobilization of the microalgae *Tetraselmis chuii* and the cyanobacterium *Spirulina maxima* (Da Costa and De França 1996).

The biomass of dead microalgae cells is also be very efficient in accumulation of heavy metals: cyanobacterium *P. laminosum* biomass immobilized in beads of polysulfone and epoxy resin was successfully used for Cu(II), Fe(II), Ni(II), and Zn(II) (Blanco et al. 1999). It was detected that the amount of biosorbed metal increased with biomass content and can be retained for several cycles of biosorption followed by acid desorption. The cells of *Ascophyllum nodosum* immobilized in hydrophilic polyurethane foam effectively adsorbed copper from the aqueous media (Alhakawati and Banks 2004). The using of *Microcystis* sp. cells for copper removal from the media gave better result than chemical adsorption with sodium polystyrene sulfonate (NaPSS) (Jang et al. 1999).

Chlorella sorokiniana immobilized on loofa sponges (Akhtar et al. 2008) was used for bioremoval of nickel, cadmium, chromium, and lead from industrial wastewater: the maximum adsorption capacity for Cd and Ni was *ca*. 192 mg g^{-1} and 71 mg g^{-1} of the immobilized biomass, respectively. The adsorption efficiency was always higher for immobilized microalgae than that estimated for suspended cells. Notably, loofa sponge itself without immobilized cells adsorbed only a small amount of this metal. The maximum amount of lead was absorbed in 5 min at pH 5, and adsorption efficiency was 96%.

Alginate-immobilized cells of *Chlamydomonas reinhardtii* were successfully exploited for mercury, cadmium, and lead removal from wastewater (Bayramoğlu

et al. 2006). The efficient adsorption of uranium ions from both freshwater and seawater samples was carried out with cells of *Chlorella* sp. entrapped in polyacrylamide gel (Nakajima et al. 1982). At pH 5.0–6.0, removal of Hg(II), Cd(II), and Pb(II) ions by immobilized microalgae was 89.5, 66.5, and 253.3 mg g^{-1} dry weight correspondingly. It was reported that immobilized microalgae are currently used for separate isolation and concentration of palladium, platinum, lead, copper, cadmium, and gold (Carrilho et al. 2003). In acidic media (pH <2), *C. vulgaris* cells immobilized on an anion-exchange resin Cellex-T effectively removed palladium and platinum (Dziwulska et al. 2004).

Conclusions Experimental evidence summarized in this review suggests that immobilization can be beneficial for industrial cultivation of microalgae. Immobilization facilitates harvesting of microalgae, elevates their stress tolerance, and provides flexibility to design photobioreactors and other cultivation and biotreatment systems. All these advantages contribute to the higher productivity of microalgae and enhance the efficiency of wastewater biotreatment. Currently, the special biofilm photobioreactors (PBR) are being developed for generation of value-added biomass and metabolites as well as for wastewater treatment. Finally, it should be emphasized that successful application of immobilized microalgae under real-life conditions entirely depends on the informed choice of the organism, optimal carrier, and immobilization technique.

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Chapter 8
Marine Microbial Biosurfactants: Ecological and Environmental Applications

K. V. Deepika, G. Mohana Sheela, and Pallaval Veera Bramhachari

Contents

Abstract The application of microbial biosurfactants in environmental technologies is gaining interest due to growing environmental concerns. Recently, immense attention is given to the environmental issues caused by synthetic surfactants owing to their toxicity and complexity in being decomposed in the environment. Microbial biosurfactants are promising because of their biodegradability, low toxicity, and efficacy in increasing biodegradation and solubilization of insoluble compounds at extreme environmental conditions. These molecules are impending substitutes for chemical surfactants in various industrial and environmental applications, such as lubrication, wetting, softening, emulsion forming, stabilization of dispersions, anti-foaming, enhanced oil recovery, as well as bioremediation of oil, heavy metals, pesticides, and organic/inorganic contaminants. The current review emphasizes a comprehensive overview on the environmental applications of biosurfactants and their role in various environmental processes.

Keywords Microbial biosurfactants · Bioremediation · Enhanced oil recovery · Environment

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8.1 Introduction

Microbial biosurfactants are amphiphilic molecules comprising hydrophilic polar moiety which can be an oligo- or monosaccharide, protein, polysaccharide, or peptide, and unsaturated or saturated fatty alcohols form the hydrophobic moiety (Rodrigues 2015). The main significant feature of surface active molecules is hydrophilic-lipophilic equilibrium which imparts both hydrophilic and hydrophobic nature to these compounds. Owing to the amphiphilic nature, biosurfactants are able to raise the surface area of water insoluble compounds as well as modify the cell surface properties of microorganisms. Biosurfactants serve as good foaming agents, emulsifiers, and dispersing agents owing to their surfactant nature (De et al. 2015). They are functionally dynamic at harsh conditions of high temperature, salt, and pH. Various properties of biosurfactants including dispersion, emulsification, de-emulsification, wetting, and foaming prove their effectiveness in remediation through biological processes and removal of metal and organic contaminants (Wu and Lu 2015). Biosurfactants are able to complex with metals and carry out elimination of heavy metals from surface soil layers in metal-contaminated sites (Sarma and Prasad 2015). Because of their surface active properties, they enhance the solubility of hydrophobic particles like pesticides accumulated in soil and water (Neitsch et al. 2016).

Bioremediation overcomes the conventional cleanup processes by emerging as novel economical method to resolve various concerns related to environmental pollution. Biosurfactants are well known as desirable alternatives to synthetic surfactants in remediation of polluted areas and oil spill treatments (Mulligan 2005) (Fig. 8.1). Biosurfactants enhance hydrocarbon degradation by increasing their bioavailability due to emulsification of hydrocarbon-water mixtures. When compared to synthetic surfactants, microbial surfactants are less toxic as well as stable and efficient in promoting bioremediation technology. Moreover, a variety of substrates including industrial wastes can be used for their production with simple experimental methods (Muthusamy et al. 2008). This chapter comprehensively discusses various environmental applications of biosurfactants in turn to provide novel approaches for the advancement of efficient and ecofriendly alternatives for the bioremediation of contaminated sites. It also discusses how the future research needs to be addressed to make biosurfactant-based remediation strategies more accessible.

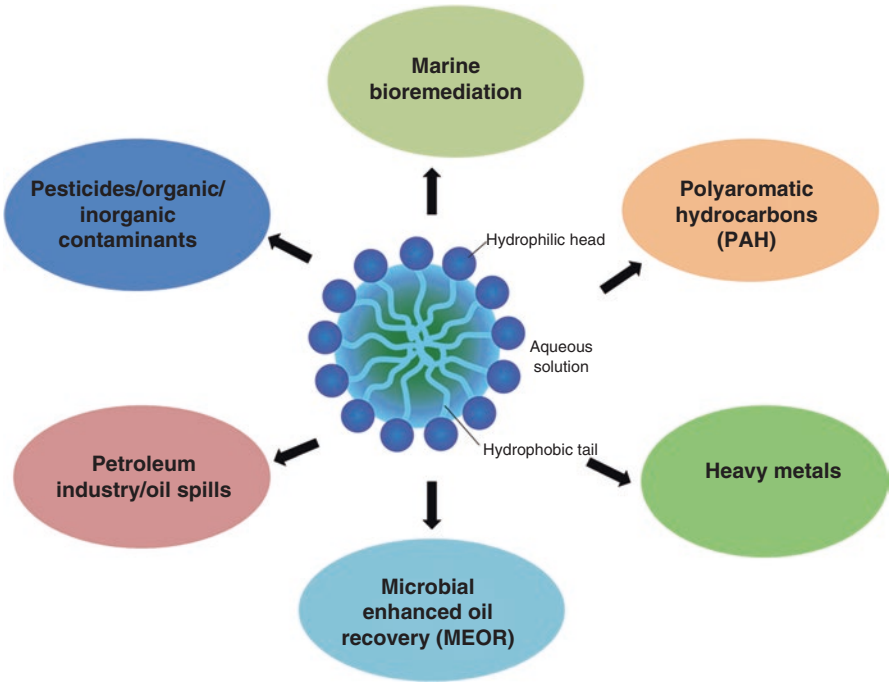

Fig. 8.1 Environmental applications of biosurfactants

8.2 Role of Biosurfactants in Marine Bioremediation

Bioremediation offers significant prospective for economical, pollutant-specific treatments to eliminate ecological pollutants from marine ecosystem. However, it involved the speeding up of native biodegradative practices in polluted areas by increasing the bioavailability of nutrients, oxygen, pH, moisture content, and existing microbial population. The presence of marine microorganisms with ability of oil degradation is well established, and previous studies established the ability of native microflora to degrade petroleum hydrocarbons soon after exposure (Swannell et al. 1996). Bioremediation experiments in marine environment vary significantly with regard to geographical region, type of oil and concentration, proportions of study area, site of oil on seashore, composition of sediments, and the analytical techniques employed to assess process of biodegradation (Swannell et al. 1996). In a study by Chakrabarty (1985), *Pseudomonas aeruginosa* SB30 produced an emulsifier which could scatter oil into small droplets which is beneficial in removing oil from contaminated beaches. Few experimental studies suggested the supplementation of additional nutrients along with the biosurfactants can enhance the process of bioremediation. This hypothesis is supported by the experimental evidences of McKew et al. (2007). Notably, they showed that combined addition of bioemulsifier,

nutrients, and oil degrading organisms like *Thalassolituus* and *Alcanivorax* reflected a synergistic effect, ensuing more degradation of n-alkanes (73%), branched alkanes (59%), and polynuclear aromatic hydrocarbons (28%) in the initial 5 days of biodegradation processes. Similarly, Nikolopoulou and Kalogerakis (2008) demonstrated the efficacy of addition of fertilizers possessing nitrogen and phosphorus to remediate oil spills (96% removal of C_{19}–C_{34} n-alkanes) in marine environments. In a recent study, Lee et al. (2018) reported some indigenous biosurfactant-producing bacteria which notably desorbed crude oil in oil-contaminated marine sediments.

8.3 Role of Biosurfactants in Bioremediation of Polyaromatic Hydrocarbons

Polyaromatic hydrocarbons can be defined as the organic pollutants primarily formed due to the incineration of fossil fuels. They are considered as one of the most hazardous contaminants owing to their perseverance, toxic, mutagenic, and carcinogenic nature (Cerniglia 1992). Physicochemical and biological factors influence the rate of biodegradation. The capability of hydrophobic compounds to be dissolved and transported into microbial cells which can metabolize them constitutes the rate-limiting step in the process of bioremediation. Noordman and Janssen (2002) investigated the efficacy of rhamnolipids for enhanced removal of phenanthrene using rhamnolipid solution (500 mg/L) and reported that removal of phenanthrene was enhanced (two–fivefold shorter time required for 50% recovery and 3.5-fold for 90% recovery) in contrast to controls. In addition, *Sphingomonas paucimobilis* EPA505 produced Alasan biosurfactant which increased the rate of phenanthrene mineralization (Barkay et al. 1999). Likewise, it was found that fluoranthene served as sole source of carbon in *Sphingomonas paucimobilis* EPA505 (Kanaly and Harayama 2000). Some marine bacteria, for instance, *Cycloclasticus spirillensus*, *Lutibacterium anuloederans*, and *Neptunomonas naphthovorans*, were previously used in improved biodegradation of PAHs in marine ecosystem (Chung and King 2001).

Similarly, Samanta et al. (2002) reported naphthalene-degrading marine bacteria including *Achromobacter denitrificans*, *Bacillus cereus*, *Corynebacterium renale*, *Cyclotrophicus* sp., *Moraxella* sp., *Mycobacterium* sp., *Burkholderia cepacia*, *Pseudomonas fluorescens*, *Pseudomonas paucimobilis*, *P. putida*, *Brevundimonas vesicularis*, *Comamonas testosteroni*, *Rhodococcus* sp., *Streptomyces* sp., and *Vibrio* sp. However, Teramoto et al. (2011) opined that bacteria in the genus *Oleibacter marinus* have key role in biodegradation of hydrocarbons. A biosurfactant from marine *Bacillus circulans* was reported to enhance the availability and biodegradation of anthracene (Das et al. 2008). Bezza and Chirwa (2015) reported improved biodegradation of used motor oil polycyclic aromatic hydrocarbon compounds up to 82% in 18 days of incubation using a lipopeptide produced by *Bacillus subtilis* CN2. More recently, biosurfactant produced by *Ochrobactrum intermedium* collected from crude oil-contaminated soil efficiently solubilized naphthalene and phenanthrene (Ferhat et al. 2017).

8.4 Role of Biosurfactants in Bioremediation of Metal-Contaminated Soils

Heavy metal pollution in urban areas and agricultural lands was caused by the activities like mining, production, and utilization of synthetic commodities (e.g., pesticides, paints, batteries, industrial waste materials). In contrast to the organic molecules, restoration of metal polluted environments is predominantly demanding as metals can be either biodegraded or biotransformed. Therefore, biological processes were considered as effective alternatives in the restoration of metal polluted environments. Marine microorganisms are found to interact with and modify the characteristics of a variety of noxious and nonhazardous metals (Haferburg and Kothe 2007). In a study, Von Canstein et al. (2002) reported efficient removal of mercury by a consortium of marine bacteria in a bioreactor. Interestingly, the ability of chelation of toxic heavy metals by bacteria of marine origin was reported in *Enterobacter cloacae* showing chelating efficiency up to cadmium (65%), copper (20%), and cobalt (8%) at 100 mg/L of metal concentration (Iyer et al. 2005). In another study, Abd-Elnaby et al. (2011) investigated a marine *Vibrio harveyi* capable of bioaccumulating Cd up to 23.3 mg Cd^{2+}/g of dry cells. Similarly, purple nonsulfur marine bacteria such as *Rhodobium marinum* and *Rhodobacter sphaeroides* were able to remove heavy metals like Cu, Zn, Cd, and Pb either by biosorption or biotransformation (Panwichian et al. 2011).

Remarkably, enhanced removal efficiency of Cd (99%) and phenanthrene (80–88%) was observed with the exploitation of lipopeptides suggesting their application in concurrent exclusion of heavy metal ions and organic contaminants (Lima et al. 2011). Likewise, Wang and Mulligan (2009) described the prospective of microbial surfactants to remove arsenic from mine tailings. In a study by Gnanamani et al. (2010), a marine *Bacillus* sp. MTCC 5514 produced potent biosurfactant which can remove Cr (VI) through bioremediation. Similarly, Asci et al. (2010) established that metal ions (91.6% of Cd and 87.2% of Zn) may possibly extract from quartz by treating with rhamnolipid. The processes promoting formation of biosurfactant metal complexes include ion exchange, precipitation-dissolution, counter-ion association, and electrostatic interaction (Rufino et al. 2011). In another study, Rangarajan and Sen (2013) evaluated the elimination of calcium, magnesium. and Fe (II) in bubble column experiments utilizing lipopeptide secreted by *Bacillus megaterium* strain of marine origin. It is notable that a surface-active compound produced by *B. subtilis* ICA56 was evidenced to be efficient in exclusion of metals such as Cu, Cr, and Zn from contaminated systems (de França et al. 2015). Yang et al. (2016) showed toxic metal removal efficiency (44.0% for Zn, 52.2% for Mn, 37.7% for Cd) of biosurfactant produced by *Burkholderia* sp. Z-90. In a recent study, Tang et al. (2018) depicted the enhanced removal of toxic metals from sludge in the electrokinetic tests by using rhamnolipid, saponin, and sophorolipid, respectively.

8.5 Role of Biosurfactants in Microbial Enhanced Oil Recovery

Microbial enhanced oil recovery utilizes microbes and their metabolic processes for the exploitation of oil from reservoirs. Microorganisms synthesize surface active compounds which decrease oil-rock surface tension by reducing capillary forces that hinder the mobility of oil through pores of rock (Banat 1995). Microbial biosurfactants assist in mobilization of hydrocarbons, emulsification, and breakdown of oil films (Perfumo et al. 2010). However, multiple effects of microorganisms such as production of gases and acids, fall in viscosity of oil, plugging by biomass growth, decrease in interfacial tension by microbial surfactants, and breakdown of large organic molecules contribute to the oil removal. Interestingly, all these factors facilitate the decrease in oil viscosity and easy recovery. MEOR is proven to be economical in comparison with chemically enhanced oil recovery, as microbes synthesize competent surface active molecules on inexpensive substrates (Sarafzadeh et al. 2014). Numerous experimental studies highlighted the application of microbial surfactants in microbial enhanced oil recovery (Bordoloi and Konwar 2009; Liu et al. 2015). Apparently, Yan et al. (2012) achieved an oil recovery of up to 91.5% from oily sludge using rhamnolipids produced by *Pseudomonas aeruginosa* F-2 in in vitro and small-scale experiments. A *Clostridium* strain NJS-4 facilitated improved oil recovery of 26.7% and 10.1% in sand pack experiments and core flood experiments correspondingly (Arora et al. 2014). Likewise, Zhao et al. (2015) demonstrated enhanced displacement of 8.33% crude oil in core flooding experiments during in situ production of rhamnolipid by *Pseudomonas aeruginosa* strain SG. More recently, Najafi-Marghmaleki et al. (2018) reported a novel *Alcaligenes faecalis*, producing 8.2% and 5.2% further oil recovery in microbial enhanced oil recovery process in a carbonate oil reservoir.

8.6 Role of Biosurfactants in Petroleum Industry and Remediation of Oil Spills

Despite being major pollutants of the environment, petroleum hydrocarbons are main energy resources. Petroleum hydrocarbons vary in their vulnerability to microbial action and usually dissociate in the subsequent order of declining susceptibility: n-alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes (Leahy and Colwell 1990). Previous studies reported that biosurfactants can be synthesized primarily on water insoluble substrates like petroleum hydrocarbons (Ward 2010). Petroleum-polluted locations have more possibility of facilitating favorable situations for microbial surfactant producers, and the related bioremediation abilities were primarily investigated (De Sousa and Bhosle 2012; Syakti et al. 2013). Commonly used physicochemical methods merely transfer pollutants from an environmental medium to a new one and generate lethal by products. Furthermore, crude

oil may not be removed entirely with physicochemical processes. Therefore, further consideration is given to natural substitutes. Microbial surfactants predominantly increase the diffusion of pollutants in aqueous phase and enhance bioavailability of insoluble substrates to microbes, thereby facilitating the subsequent elimination of contaminants in biodegradation. Lai et al. (2009) demonstrated that microbial surfactants assisted in enhanced total petroleum hydrocarbon removal employing 0.2 mass% of rhamnolipids (23%) and surfactin (14%) than chemical surfactants Tween 80 (6%) and Triton X-100 (4%). An efficient microbial consortium was composed by Deppe et al. (2005) using arctic bacteria like *Agreia*, *Marinobacter*, *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter*, and *Shewanella* for dissociation of crude oil and its components. Most commonly reported oil-degrading bacterial genera include *Acinetobacter*, *Marinococcus*, *Methylobacterium*, *Micrococcus*, *Planococcus*, *Nocardia*, and *Rhodococcus* (Sakalle and Rajkumar 2009).

In a study by Batista et al. (2010), *Candida tropicalis* secreted a biosurfactant which removed motor oil from sand with removal efficiency of 78%–97%. Likewise, Luna et al. (2013) reported a Lunasan, secreted by *Candida sphaerica* UCP 0995 which separated 95% of motor oil from sand. Several studies reported the prospective uses of bioremediation techniques in mangrove soils polluted with petroleum hydrocarbons in Australian continent (Burns et al. 2000) and Brazil (Brito et al. 2009). More recently, Silva et al. (2014) evaluated the potential of *Pseudomonas cepacia* CCT6659 biosurfactant for removing oil from contaminated beaker walls and noticed an elimination rate of 80% suggesting the use of this microbial surfactant in cleaning of oil storage tanks. *Marinobacter* sp. producing a novel biosurfactant which function as biodispersant to combat marine oil spills was isolated by Raddadi et al. (2017).

8.7 Role of Biosurfactants in Bioremediation of Recalcitrants

Over the years, pesticides were used for the control of pests and disease vectors in agriculture. Nevertheless, improper utilization of these compounds resulted in environmental pollution due to their toxicity, bioaccumulation, instability, and persistence. Notably, the ecofriendly biological methods including natural attenuation, immobilization of cells, biosurfactant production, and bioaugmentation were proved to be affective in combating adverse effects of these compounds (Benimeli et al. 2018). Bioremediation strategies include in situ production of surface active compounds by microbes to enhance the bioavailability of chemical pollutants (Gnanamani et al. 2010; Wattanaphon et al. 2008).

Microbial biosurfactants produce complexes with pollutants bound to the soil particles and increase their availability through desorption. Strikingly, these hydrophobic pesticides become stable within the biosurfactant micelles which sequentially favor their solubility following exclusion in the washing process. Several studies highlighted the crucial role of actinobacteria as the potential candidates to remove organic and inorganic pollutants. A glucolipid-type biosurfactant produced

by *Burkholderia cenocepacia* BSP3 evidently improved solubilization of pesticides like methyl parathion, ethyl parathion, and trifluralin suggesting its role in environmental remediation (Wattanaphon et al. 2008). Kumar et al. (2008) reported biodegradation of α-endosulfan (73%) and β-endosulfan (75%) using a novel strain *Arthrobacter.* In another study, Kolekar et al. (2013) showed the biodegradation of atrazine by *Rhodococcus* sp. BCH2, which can use this as carbon and nitrogen source. Similarly, Khessairi et al. (2014) demonstrated the degradation of pentachlorophenol using *Actinobacterium janibacter* sp. AS23. Sagarkar and his coworkers (2016) reported the degradation of S-triazine by *Arthrobacter* sp. strain AK-YN10. More recently, a *Pseudomonas* sp. strain B0406 was isolated by García-Reyes et al. (2017) which increased the solubility of endosulfan and methyl parathion. A glucolipid-type biosurfactant noticeably enhanced pesticide solubilization suggesting its role in environmental remediation.

8.8 Conclusions

The current review emphasizes various environmental applications of biosurfactants. A variety of metals and chemical contaminants can be removed using biodegradation processes. Exploitation of biosurfactants in field applications does not require purity grade; thus purification costs may possibly be more moderate. Nevertheless, efficiency of the process may vary with different structural characteristics of biosurfactants. Future research investigations have to be focused on comparison of effectiveness of various biosurfactants in removal of contaminants. More information is requisite pertaining to the interactions of biosurfactants with pollutants, relation with structure of biosurfactant, pollutant removal efficiency, and geochemical features of polluted area. Despite this, we still need to obtain more knowledge on the aspects influencing removal rates of pollutants by various biosurfactants. Merely a few marine biosurfactant-producing microbes have been explored till date owing to the hurdles related to their isolation and growth. Other promising isolation methods including culture-independent methods and metagenomic techniques can be explored for the detection of novel biosurfactant producers from extreme environments.

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Conflict of Interest We declare we have no conflict of Interest

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Chapter 9
Effect of Biochar Addition to Soil on Plant Quality

Abdul Bakrudeen Ali Ahmed, Mohaddeseh Adel, Ali Talati, Vijay Lobo, and V. D. Seshadri

Contents

Abstract Thermal decay of organic compositions under a non-oxygen condition causes the production of biochar. Biochar is an organic matter used as a soil amendment, with the intent to improve soil properties. Biochar is a valuable source to produce medicinal compounds due to its fewer side effects and economically

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efficient properties. Biochar feedstock is mostly obtained from farm wastes, and its nature, type, and origin determine the quality and composition characters of biochar. Besides, adding biochar to soil improves the soil retention, increases its nutrition concentration, has potential to improve the poor soil quality, and improves the growth rate and yield of plants. In this short review, we reviewed the effects of biochar on nutrition value of medicinal plants and crops. However, the composition of biochar and its nutrient availability are studied. Moreover, the environmental solutions of using biochar and the benefits biochar provides such as higher crop yields, increased fertilization efficiency, and more plant growth besides preventing nutrition discharge into the groundwater were reviewed. In addition, the production of a secure habitat for favorable microbial activity, which causes a boost in soil nutrients, besides other biochar properties, characters, and impacts on soil, plant growth, and environment is discussed.

Keywords Biochar · Feedstock · Medicinal plant · Nutritional value · Crop yield

9.1 Introduction

Biochar is produced by the thermal decay of organic compositions under a non-oxygen condition and at a comparatively low temperature. In addition, pyrolysis uses biomass baking in the absence of air to take off the volatile gasses and keep the carbon behind. Hence, besides biochar, the bio-energy produces in the form of gas and oil. This energy considers as a form of new and renewable fuel. Keeping the environmental solution aside, biochar is a source of benefits for farmers, such as production of higher crop yield, elevation in fertility, enhancement in soil quality, reduction of the groundwater contamination by pollutants, and heat production, besides selling the carbon credits in the carbon markets (Milne et al. 2007; McHenry 2009).

The term 'biochar' first appeared in the modern scientific literature in a paper presented by Harshavardhan Bapat and Stanley E. Manahan at the 215th National Meeting of the American Chemical Society in 1998. They won first prize for that paper from the ACS division of Environmental Chemistry in the category of poster presentations: Gasification of Hazardous Waste and Mixed Wastes on a Biochar Matrix. Commonly, the biochar particles settled with low density and high porosity that makes it to keep more water and air; hence the soil bulk density reduces (Downie et al. 2009). Applying biochar causes an increase in water holding capacity of soil, soil pH, porosity, and stabilization of soil organic carbon by elevating its aggregation and decreasing its tensile strength and bulk density (Cao et al. 2009; Spokas et al. 2009; Zheng et al. 2010; Abel et al. 2013; Liu et al. 2016).

While soil is amended by biochar, the soil water holding capacity increases because of soil bulk density, and it simulates growth in root and enhancement in microbial activity of the soil (Major et al. 2010; Zheng et al. 2010). Another study, addition of biochar to a highly productive Clarion loam reduced soil bulk density (Laird et al. 2010). Tammeorg et al. (2014) also showed that by biochar application, the available water of the plant at the top 20 cm of soil during the first year is higher and the soil bulk density decreased during the second year. It was also reported that application of biochar reduces soil tensile strength, which leads to reduction in tillage costs (Vaccari et al. 2011).

9.2 Medicinal Plants and Application in Terms of Biochar

Medicinal plants have a long history of human life and been identified and used for centuries. Some kind of plants produces and synthesizes chemical compounds which are important in performing biological functions. Medicinal plants are important effective sources against different kinds of infectious agents. Medicinal plants are more available resources and have fewer side effects. They are also economic and have antimicrobial properties. Medicinal plants are among the precious resources in developing antibiotics. Despite the value of herbs for flavoring, aromatic, and medicinal properties, the modern age production of herb excels their importance.

The mishandle of antibiotics caused the emergence of some sort of pathogenic strains from bacteria that are antibiotic resistant. Accordingly, after an extended period of antibiotic consumption, the bacteria become resistant to it and behave less effective. Hence, the pharmaceutical companies that focus on manufacturing new generation of antibiotics strive in knowing how to handle the bacterial strains which are resistant to antibiotics.

9.3 Biochar Effects on Crops and Medicinal Plants

A carbon-based porous material has proven to provide higher crop yields, enhancing soil health and maintaining nutrients in the soil and preventing their discharging into the groundwater. Physical and chemical nature of biochar has a special ability for holding and attracting moisture, nutrients, and agrochemicals, even those which are retaining difficult such as nitrogen and phosphorous to hold in soil. This is because nitrogen always tends to run-off from regular soils and is causing ecosystem balance disruption in streams and riparian areas.

In addition, biochar's massive complex pore structure and surface area provides a safe habitat for fungi and microorganisms. For a greater nutrient uptake by plants, some specific fungi can form a symbiotic relationship with the plant root fibers.

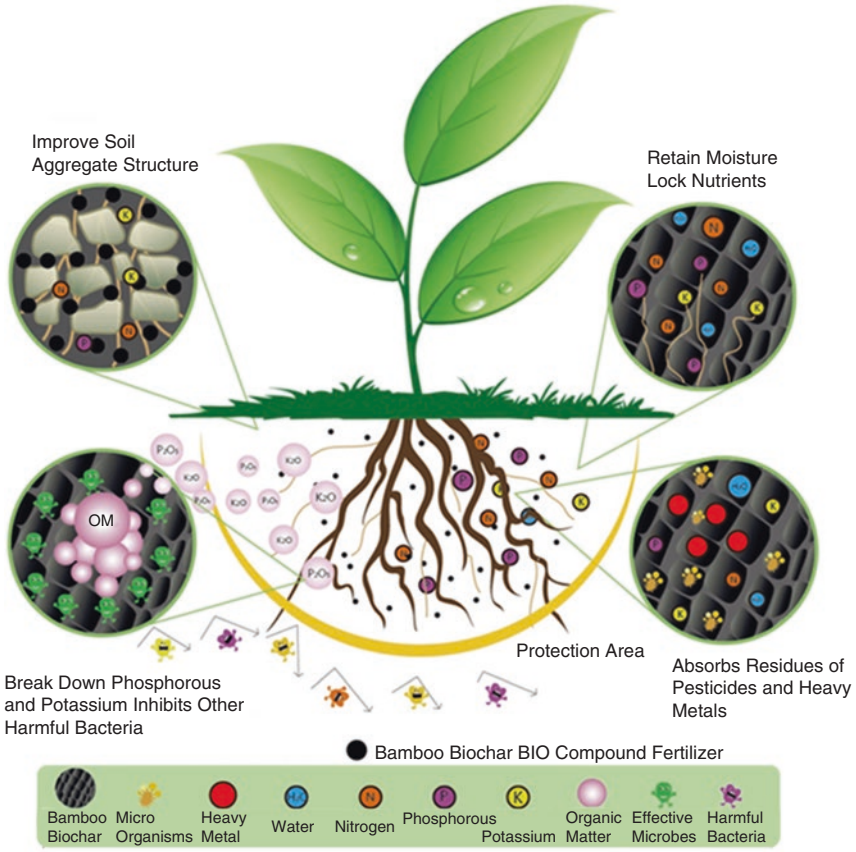

Fig. 9.1 Biochar effects on crop development

Some researcher has proven that biochar can hold gasses which will reduce nitrous oxide and carbon dioxide emissions by 50–80%. However NO_2 is an important greenhouse gas and has 310 times more potent than CO_2 (Schmidt 2012; Karhu et al. 2011). These are some of beneficial effect of biochar on crops and medical plants (Fig. 9.1).

9.4 Composition of Biochar

Most of the feedstock used in biochar production is obtained from farm yield wastes, such as grass and corn stover, and the diseased trees which were removed by the Forestry department. In addition, some other feed stocks used for biochar synthesis are as follows: livestock and poultry wastes, wood processing residues, wastewater, municipal solid wastes, and biosolids (Lehmann and Joseph 2015; Duku et al. 2011).

Fig. 9.2 Different type of feedstock used in biochar production

The nature, type, and origin of the feedstock affect the quality and composition characters of biochar such as surface area, porosity, moisture content, particle size distribution, surface area, and pH (Downie et al. 2009; Rajapaksha et al. 2014; Zhang et al. 2008; Enders et al. 2012). Pyrolysis possesses various advantages that one of them is the wide variety of feedstock materials that are used, such as bioenergy crops, e.g. Karnal grass, hybrid poplar, and switch grass. These feedstock materials sequester carbon through root biomass decaying within the soil layer (Tilman et al. 2006). Nguyen and colleagues suggested that biochar made from crop residues has a higher quality compared to biochar made from wood residues due to higher decrease in NH_4^+ and NO_3^- in crop residues (Nguyen and Tran 2019) (Fig. 9.2).

9.5 Nutrient Availability and Leaching

Griffin et al. (2017) who researched about the effect of biochar addition from walnut shell on nutrient level of the soil found out that biochar most likely increases the soil nutrient concentrations through direct and indirect mechanisms. There are many studies which shows that biochar due to its higher cation exchange capacity plays a vital role in the improvement of soil nutrient retention (Rajkovich et al. 2012; Sun et al. 2014; Iftikhar et al. 2018).

9.6 Comparison of Plant and Organ Development

Enhancing crop yields and improving plant growth can be obtained by adding biochar to the poor soil, increasing the food production and conserving an ecological balance in areas with poor and empty soil, minimal organic resources, and

inadequate water and/or access to agrochemical fertilizers. However, each depleted soil has a different reaction to the same biochar and, to observe the result, can take up to a year. Some studies have shown the biochar potential which can help to increase crop yields up to four times on poor soils with low carbon content. Yang and coworkers reported a significant rice growth, plant height, and root length promotion and growth of rice seedlings by addition of biochar-extracted liquor (Yang et al. 2019).

Another study by Liu and colleagues represented a significant increase in crop yield by mitigating N_2O and CH_4 emissions. They stated that biochar enhances soil fertility by increasing the availability of water and soil nutrients (Iftikhar et al. 2018; Liu et al. 2019).

Fertilizers and biochar have a synergistic in enhancing crop yield; for instance, maize productivity can raise more with the mixture of biochar and fertilizer application compared to fertilizer alone in acidic soil in Indonesia (Yamato et al. 2006). Biochar has an ability to improve crop yield under unfavorable conditions such as salinity and drought (Thomas et al. 2013; Haider et al. 2015). For example, biochar enhanced the permanent wilting point (Abel et al. 2013; Cornelissen et al. 2013); however, the quantity of water preserved at field capacity has been improved compared to the water held at eternal wilting point and increased plant available water (Fig. 9.3).

Fig. 9.3 Biochar without fertilizer compared with plain soil

9.7 Nutrition and Quality

Biochar has the ability to improve soil fertility resulting in higher crop yields. Biochar applied in finely ground form to the soil causes the gathering of beneficial fungi and bacteria due to the wide surface area and complex pore structure of biochar which eventually help the plants absorb nutrients from the soil. Hence, biochar can provide a secure habitat for favorable microbial activity that is important for crop yields and production to flourish.

Biochar appeared to improve the uptake of methane and reduce the soil emissions of nitrous. For example, soil emissions of nitrous oxide reach about half compared to the greenhouse gas emissions from corn production (one quarter of the emissions from corn ethanol production). Then, along with corn-ethanol production, minimizing 50% of nitrous oxide emissions would substantially improve the carbon footprint of corn production.

Most parts of the world suffer from soil acidity (low pH). Mainly, correction of soil acidity is by applying large amount of lime to increase the pH level. However, in acidic soil, biochar has the capability to lower soil acidity. Biochar can directly maintain soil nutrients through the negative charge that develops on its surfaces and can buffer acidity in the soil (Tenenbaum 2009).

Due to biochar's potential to boost the availability of plant nutrients, soil nutrient retention is enhanced which effects moderation of fertilizer needs bringing down the cost of producing the crop. In addition, char-amended soils have lower the run-off of phosphorus into surface waters and leaching of nitrogen into groundwater (Tenenbaum 2009). Biochar increases the soil pH, which increases the production of N_2 rather than N_2O, improves soil aeration and more O_2 uptake, and completes denitrification by increasing dissolved organic carbon (Liu et al. 2019).

9.8 Cultivation and Yields

By using biochar in the soil, physical and chemical properties of the soil which increase the production and crop yield may be maintained. In addition, soil fertility is reinstated by motivating microbial activity base on organic matter, fixing atmospheric nitrogen, and preserving soil from erosion, salinity, and acidity. Biochar also assists in controlling pests, insects, and soil-borne infections and illnesses and also controls weeds; for example, repeated wheat culture grows wild oats and phallaris infestation. Similarly, culturing berseem continuously motivates chicory infestation, e.g. kasani, but an alternate cropping of berseem and wheat helps in controlling kasani as well as oats and phallaris.

Biochar also inhibits or limits periods of peak demands of irrigation water. In crops desiring high irrigation, if it is followed by light irrigation, it will not influence or degenerate the soil physical condition (Tenenbaum 2009). The potential biochar have ability to remove the toxic elements from plant and helps to improve the plant

Fig. 9.4 The field observation on corn grew poorly, tasseled weak, and formed incomplete ears of irregular seeds without using fertilizer. However, the area with charred residue grew full-size corn with vigorous tassels, heavy silks, and full, well-formed ears

growth with health (Liu et al. 2019). It was also stated that applying biochar in dry lands mitigates global warming and enhances crop production compared to the paddy fields (Liu et al. 2019) (Fig. 9.4).

9.9 Conclusion

- Biochar is a carbon base organic matter that is used for soil amendment in increasing crop yields and production.
- Biochar-produced plants used for preparing medicinal plants are important available sources with fewer side effects.
- Most of the feedstock used in biochar production is obtained from farm yield wastes, such as grass and corn Stover.
- The addition of biochar to soil enhances the soil retention and nutrition concentrations.
- It helps prevent nutrition discharge into the groundwater and enhancement in soil health, crop yield, and plant growth.
- Biochar reduces soil emissions of nitrous oxide and enhances the methane uptake and soil pH.
- Biochar motivates microbial activity base on organic matter, fixing atmospheric nitrogen, and preserving soil from erosion, salinity, and acidity.

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