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Bioplastics for Sustainable Development

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Dedicated to our beloved supervisors . . .

Preface

Plastics are unquestionably the world's most versatile polymer that can be turned into anything and everything for human benefit at a very low cost. But to tackle the pollution generated from discarded plastics is very difficult. Whole marine and soil ecosystems are literally drowning in plastics. The worst part of these polymers is that they are synthesized from expensive and nonrenewable resources and oil and petroleum products. Therefore, there is a requirement for materials that are degradable, recyclable, and eco-friendly. This brings the picture of bioplastics to the world that are synthesized from natural materials. Bioplastics are synthesized by many microbes and accumulated inside the cells under stress conditions as a storage material. Microbial enzymes also play a crucial role in their degradation.

Ironically, all bioplastics do not show the same fate and leave toxic residues or plastic fragments behind that are unsuitable for composting. Bioplastic waste makes a difference to the environment only when they are buried in landfills. Moreover, some require industrial composting facilities to break down and the majority do not have access to this. When these wastes are not left in landfills to degrade, then these are incinerated and thereby give similar environmental impact as conventional plastics do. These "green" plastics, which are supposed to be a great alternative as a replacement, in fact are not better for the environment also increase the level of pollution on land and in water. So, there are benefits and drawbacks of these bioplastics at the same time. Now, it is up to the producer and consumer to decide how they deal with this significant but controversial issue.

This book will be a comprehensive reference in the most progressive field of bioplastics and will be of interest to professionals, scientists, and academics related to biotechnology and polymer science. This book covers 23 chapters that provide an updated knowledge of bioplastics and biodegradable plastics which is currently a burning topic for today's need. It will clarify the accurate difference between biodegradable, recyclable, and bioplastics that are actually the same for a common man. The chapters highlight the potential impact of bioplastics and its significant applications in various medical and biotechnological sectors. Alternate cheap substrates like agro-wastes and fruit waste are also explored for the production of these biopolymers. Other chapters explain the novel source and technologies adopted for the production of bioplastics, which are continuously added to the literature reports day by day. Some chapters also discuss the role of these bioplastics in food packaging, agricultural and horticultural applications, also in bioremediation. These chapters also present future perspectives for the development of specific and more active polymers for sustainable environment.

In conclusion, this book is an updated reference in the most progressive field of bioplastic polymer that will be useful for professionals, scientists, and academics related to this field. Last but not least, we would like to express our deepest sense of gratitude and regards to our family for their love and moral support, which helped us to complete this comprehensive book. We would also like to thank all the authors who have eagerly contributed their chapters to this book. Finally, we also express our sincere gratitude to Springer for providing us this opportunity.

Hail, Saudi Arabia Mohammed Kuddus
Lucknow, Uttar Pradesh, India Roohi
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About the Editors

Mohammed Kuddus is a Professor and Chairman of the Biochemistry Department at the University of Hail, Kingdom of Saudi Arabia. He has previously served at the Department of Biotechnology, Integral University, Lucknow, India (2007–2012). Holding a Ph.D. in enzyme biotechnology from SHUATS, Allahabad, India, Dr. Kuddus's main research areas include enzymology, biochemistry, and microbial biotechnology. He has more than 15 years of research and teaching experience in biochemistry, biotechnology, and enzymology. He is the editor of four books and has published more than 65 research articles in esteemed international journals, along with 20 book chapters. He is a member of various national and international scientific societies and organizations, e.g., the Indian Science Congress Association and the Asian Council of Science Editors. He also serves as an editor/editorial board member or reviewer for more than 30 peer-reviewed international journals. He has been awarded Young Scientist Projects from the Department of Science and Technology, India, and the International Foundation for Science, Sweden.

Roohi is an Associate Professor at the Department of Bioengineering, Integral University, Lucknow, India. Her main research interests are in enzyme biotechnology, especially protein biochemistry and microbial biotechnology. She has more than 12 years of research and teaching experience and has published more than 35 research articles in peer-reviewed international journals and authored/coauthored numerous book chapters. She is a member of various international scientific societies and organizations, e.g., the International Society for Research and Development and European

Biotechnology Network. She also serves as a reviewer for more than ten reputed international journals. She has been recognized with an Early Career Research Award (ECRA) and Project Grant from the Science and Engineering Research Board (SERB), Department of Science and Technology (DST), India.

Microbial Production of Bioplastics: Current Trends and Future Perspectives

1

Nupur Ojha and Nilanjana Das

Abstract

Petrochemical plastics are nonbiodegradable synthetic organic polymers. It has various applications and become an essential part of our daily lives. However, the improper disposal of plastics results in the deaths of millions of animals annually and the reduction of soil fertility and also causes severe environmental pollution. The vast consumption and accumulation of plastics have become one of the major problems throughout the world. In response to these problems, there has been considerable interest in the development and production of biodegradable microbial bioplastics which can serve as a potential alternative to petrochemical plastics. Polyhydroxyalkanoates (PHAs) are microbial bioplastics that belong to a family of biopolyesters, primarily composed of R-3-hydroxyalkanoic acid monomers unit. Wide varieties of microorganisms have been reported to synthesize polyhydroxyalkanoate (PHA) and its copolymers as intracellular inclusion under carbon-rich and other nutritional limiting conditions. PHA and its copolymers have attracted researchers and industries because of their potential use as biodegradable and biocompatible thermoplastics. It has remarkable applications in the field of tissue engineering, drug delivery, pharmaceutical, and packaging industry. The development of microbial bioplastics and their products would help to maintain the sustainability of the environment and to reduce the emission of greenhouse gases. This chapter is focused on the updated information on microbial bioplastic (PHA) production and its progress in biotechnological and other industrial applications.

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Keywords

Biodegradable · Biosynthesis · Carbon sources · Microorganisms · Microbial bioplastics · Polyhydroxyalkanoates (PHAs)

1.1 Introduction

Polyhydroxyalkanoates (PHAs) belong to a class of the polyester group, the only bioplastics which are completely synthesized from a wide variety of microorganisms under unbalanced growth conditions as a mechanism to store excess intracellular carbon and energy reserve material (Koller 2020). The microbial enzyme PHA depolymerase was responsible to catalyze polyhydroxyalkanoate (PHA) granule degradation to produce a significant amount of nutrients and energy under nutrients and energy limiting conditions for microbial cell survival and metabolism (de Paula et al. 2019). The most important characteristic feature of the PHA group is that these polyester groups are consisting of monomer units of hydroxy acid (HA) which are linked together by ester bonds. PHA has been broadly categorized into three types of PHA, viz., short-chain-length (SCL), medium-chain-length (MCL), and long-chain-length (LCL) PHA, based on the total number of carbon atoms existing in the side-chains of the PHA monomer units (Ojha and Das 2018). Monomers with up to five carbon atoms are present in the SCL-PHA type. Monomers with 6–14 carbon atoms are present in the MCL-PHA type (Koller 2018) and mostly mechanically, structurally, and thermally diverse than SCL-PHA type of polymers. However, due to the lack of efficient MCL-PHA-producing microorganisms and the necessity of costly fermentation strategies for commercial PHA production, investigation on MCL-PHA is limited (Sathiyanarayanan et al. 2017). The monomers with more than 14 carbon atoms belong to LCL-PHA-type PHA (Koller 2018; Roja et al. 2019). These microbial bioplastics have met worldwide interest and considered an alternative material for conventional plastics because of their biodegradable, biocompatible, and thermoplastic nature (Mannina et al. 2020). PHA has excellent physico-chemical properties that warrant its commercial exploitation in different applications covering from bioplastic films for crop protection, biodegradable disposable bottles, bioimplant materials, fabrication of bone marrow scaffolds orthopedic pins, sutures, adhesion barriers, stents, repair patches, swabs, drug delivery carriers, biodegradable food packaging bags, automotive, infrastructure, and aerospace to military applications (Roja et al. 2019).

Polyhydroxybutyrate (PHB) is the first microbial bioplastic that was recognized in the bacterium *Bacillus megaterium* (Mudenur et al. 2019). However, it has limited medical applications due to brittleness, limiting processing malleability, ductility, hydrophilicity, and low mechanical and structural properties (Bhatia et al. 2019a, b). To improve the material properties of PHB, various studies were investigated on the blending of PHB with other monomer units such as three hydroxyhexanoate (3HHx), three hydroxyvalerate (3HV), three hydroxyoctanoate (3HO), or four hydroxybutyrate (4HB) to formulate thermally and mechanically improved

3-hydroxybutyrate (3HB)-based copolymers (Bhatia et al. 2019a). Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV), a copolymer of PHA, has attracted the research interest because of its ability to provide a precursor for the synthesis of the copolymer in favourable condition and because it provides better physical properties as compared to PHB (Jeon et al. 2017). The copolymer hybrid P (3HB-*co*-3HV)-ascorbic acid, obtained on the addition of functional groups of ascorbic acid into P(3HB-*co*-3HV), revealed a significant improvement in the mechanical properties of P(3HB-*co*-3HV) copolymer (Bhatia et al. 2019c). In addition, the hybridization of more than one monomer unit of 3HB, 3HV, or 3HHx leads to the development of poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) P(3HB-*co*-3HV-*co*-3HHx) terpolymer with unique and enhanced properties (Jung et al. 2019a).

Recent research has shown that microbial bioplastics (PHA) exhibit severe bacterial infection, weak oxygen barrier, low strength, and poor heat resistance in packaging applications (Xu et al. 2020). These drawbacks were overcome with the addition of active agents such as antimicrobial, antioxidant, and oxygen-scavenging with PHA to control the microbial growth and preserve the desirable quality in food packaging applications (Xu et al. 2020; Mukheem et al. 2018). Besides, a novel approach of nanotechnology (i.e., the reinforcement of polymeric and metallic nanoparticles, nanoclusters, or nanotubes onto the PHA copolymers) to improve the biomedical and packaging applications has been attracted the researchers in this field (Sun et al. 2018). The multifunctional and robust PHA nanocomposites were reported by Xu et al. (2020), through compounding PHA with long-alkyl-chain quaternary salt (LAQ) functionalized graphene oxide (GO-g-LAQ) to improve the gas barrier, heat resistance, and inherent antibacterial performances. Despite having comparable characteristics to synthetic plastics, extensive use of PHA is still hampered because of its high production cost (Jung et al. 2019b; Sabapathy et al. 2019). Several biowastes such as agricultural, fruit-vegetable wastes, industrial waste products, waste frying oils, dairy wastes, and volatile fatty acids derived from food wastes have been investigated as potential carbon sources for microbial PHA production (Bhatia et al. 2019b). These waste carbon sources could convert the production of PHA into a cost-effective and eco-friendly process (Amaro et al. 2019). The effect of different fermentation processes, stress conditions, and strain improvements on the yield of PHA has also been reported to enhance PHA production (Obruca et al. 2018).

This chapter is the compilation of research updates on PHA production from microbial sources, involvement of enzymes, different classes of PHA, its properties, new strategies to incapacitate the challenges connected with the enhanced and cost-effective production process, commercially available PHA, limitations, related improvements (polymeric nanocomposites) and its potential applications available till date. Research prospects discussed in this chapter will encourage future researchers to focus on new technological attempts towards the microbial production of bioplastics.

1.2 Biosynthesis of Microbial Bioplastics

Plastics which are synthesized from microorganisms are known as microbial bioplastics. PHAs are the only microbial bioplastics that are entirely synthesized from a wide variety of microorganisms. PHA biosynthesis is accomplished by microorganisms grown in an aqueous production medium under rich carbon sources such as starch, glucose, sucrose, fatty acids, and other limiting nutrients (nitrogen and mineral salts) at 30–37 °C under atmospheric pressure (Rivera-Briso and Serrano-Aroca 2018; Muhammadi et al. 2015). Under such unfavorable growth conditions, intracellular hydrophobic PHA granules of carbon and energy are produced as byproducts, instead of major ones. Accumulated PHA does not play a vital role in the cell growth of the PHA-producing microorganisms under limiting nutrient conditions. However, the accumulated PHA helps in the survival of PHA-producing microorganisms under unfavorable environmental conditions. Therefore, microbial PHAs are considered secondary metabolites which are produced by polymerization of (R)-3-hydroxybutyryl-CoA molecules through PHA synthase leading to the PHA granules formation. PHA granules consist of a hydrophobic core surrounded by phospholipid and various additional and regulatory proteins as shown in Fig. 1.1

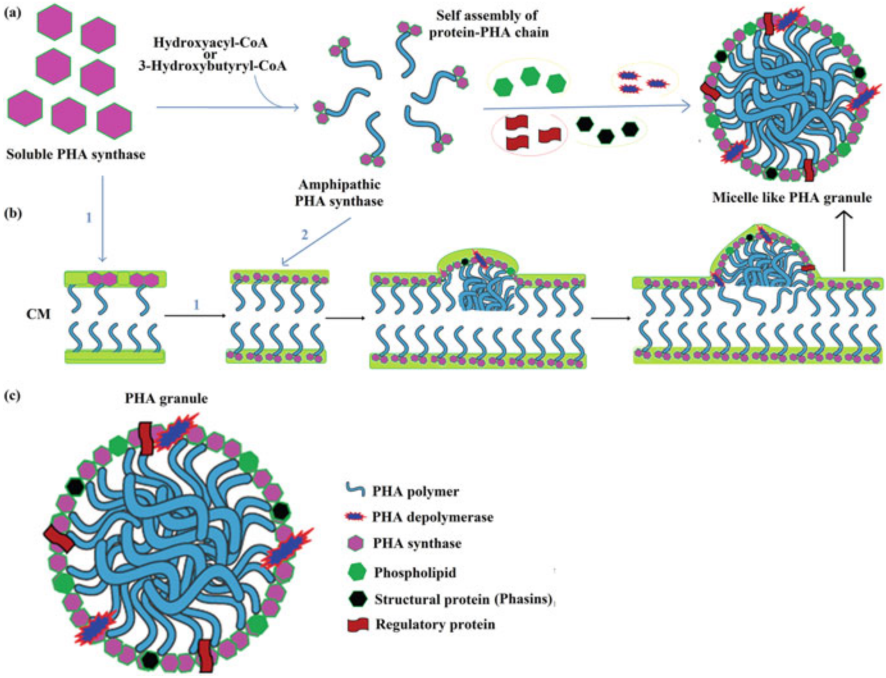

Fig. 1.1 Schematic models for PHA granule formation (self-assembly) within the cytoplasm of PHA-producing microorganisms. (**a**) In vitro assembly process. (**b**) In vivo assembly depicting two possible routes 1 and 2. CM, cytoplasmic membrane. (**c**) The ideal PHA granule surrounded by phospholipids membrane and crystalline protein

(Shrivastav et al. 2013). The accumulated PHAs are degraded by intracellular PHA depolymerases and metabolized as carbon and energy source once the supply of the limiting nutrient is reestablished (Shrivastav et al. 2013).

1.2.1 In Vitro Synthesis of Microbial Bioplastic Granules

In vitro microbial bioplastic (PHB) synthesis and self-association of spherical granules by purified PHA synthase and substrate were first reported by Gerngross and Martin (Fig. 1.1a). This study demonstrated that all the vital features needed for self-organization of the PHA chain into spherical granules were possessed by PHA synthases. The findings were further supported by the formation of in vitro synthesis of PHA by purified PHA synthases from microorganisms, such as *Cupriavidus necator*, *Pseudomonas aeruginosa*, *Allochromatioum vinosum*, and *P. oleovorans* (Rehm et al. 2001; Qi et al. 2000; Jossek and Steinbüchel 1998).

1.2.2 In Vivo Synthesis of Microbial Bioplastic Granules

Microbial bioplastic (PHA) inclusion formation has been illustrated in two models: (1) the micelle model and (2) the budding model as shown in Fig. 1.1. These models reflect the precise position of PHA synthase and to some point the phasin protein on the surface of the polyester granule. The micelle model facilitated the in vitro PHA granules alignment and the shirking of membranes (Fig. 1.1b). However, scanning and transmission electron microscopic investigations revealed the presence of membrane-like substances surrounding the whole PHA granules. Thus, the isolated PHA granules supported the confirmation for the budding model. A new model for the formation of PHA granule demonstrated that the evolving granules evolved only from the center of the cell at unidentified mediation elements (Fig. 1.1) (Tian et al. 2005). The atomic force microscopic analysis showed macro structures (35 nm) on the outward of PHB granules synthesized in *C. necator* cells which might act as synthesis-degradation centers (Dennis et al. 2003). Furthermore, in vivo granule formation of PHA as well as subcellular localization were observed by monitoring the green fluorescent protein (GFP)-labeled PHA synthase using fluorescence microscopy analysis (Peters and Rehm 2005). In this study, granule formation starts at the cell poles supporting the budding model. Overall, using GFP-labeled PHA synthase in vivo findings supported the budding model by focusing granule formation close to the cytoplasmic membrane assembled around the cell poles (Fig. 1.1b).

1.2.3 Morphology of Microbial Bioplastic Granule

Microbial bioplastics or PHAs are accumulated as light-refracting distinct granules inside the microbial cell. The light fluorescent Nile blue A or the Nile red stains and Sudan black B are used to stain PHA granules. These accumulated granules split the biopolyesters from the cell lumen without changing the osmotic pressure of the cell. Depending on the different species, the size and number of granules per cell differ. In *A. eutrophus*, a diameter range of 0.2–0.5μm was observed in almost 8–13 granules per cell, whereas in the case of *P. oleovorans*, roughly one or two large granules were investigated (Muhammadi et al. 2015). PHA granules are generally globular which are enclosed by a phospholipids membrane parting with two crystalline protein layers. Crystalline protein layers are composed of the intracellular PHA depolymerase, amphipathic phasing proteins, PHA polymerases, PHA-specific additional proteins, and regulator proteins as shown in Fig. 1.1c. The coating membrane of PHA granules is about 2 nm thick, containing 2% protein and 0.5% lipid, of the granule weight. PHA polymerases are existing in the cytosol in an inactive form and are only activated when they are confined on the granule's surface. The intracellular PHA depolymerases are linked to the granule surface, which is required for the utilization of the gathered PHA. Phasins are the principal proteins having a relatively small molecular size among all the proteins associated with PHA granules (Muhammadi et al. 2015). Based on few extensive studies, it has been demonstrated that phasins are connected with the structural function of PHA granules such as coating, stabilizing the granules being attached to the PHA core of granules by activating genes or non-covalent bond or enzymes involved in PHA synthesis. Biosynthesis of PHA is promoted by phasins and their replica number has an effect on the size of PHA granules. The phasins are performing as an obstacle between the biopolyester and other cellular components. It prevents individual granules from conjoining and adhering with other granules. The report says that engineered *R. eutropha* strain exhibited low PHB accumulation and retain only one solo big granule. This single granule formation is because of the coagulation of individual granules that occur on the open surface where hydrophobic molecules of PHA get in contact (Fig. 1.1). PHA granule-associated phasin (*phaP*) have a shielding role to diminish the inactive attachment of cytosolic proteins. Imitation of the self-association process exhibited that phasins might influence the kinetics of granule foundation by dropping the lag phase (Jurasek and Marchessault 2004; Muhammadi et al. 2015). Many PHA-specific regulators such as a DNA-binding regulatory protein (*phaR*) from *C. necator and Paracoccus denitrificans* and a nonenzymatic bifunctional protein (*phaF*) from *Pseudomonads* were capable of binding non-covalently with both PHA granules and DNA. Besides, *phaD* that is not related to PHA granules was recognized as an effective regulator protein for affecting the synthesis of MCL-PHA. The cytosolic levels of these repressor proteins slow down the accumulation of PHA granules. *PhaI* and *PhaS* are additional proteins with unknown functions, found in *Pseudomonads* (Muhammadi et al. 2015).

1.3 Mechanism and Enzymes Involved in the Synthesis of Microbial Bioplastic

Over the decades, the biosynthesis of PHA has been extensively studied by researchers. Biosynthesis of PHA takes place in the cytosol of the microbial cell by different enzymatic reactions initiated from acetyl-CoAs catalyzed by substrate-specific PHA synthases (Table 1.1). Most of the bacterial strains can produce SCL-PHAs and MCL-PHAs. So far, eight pathways/mechanisms have been investigated that are involved in the synthesis of microbial bioplastics (PHAs) (Chen 2010). The three key enzymes, viz., β-ketothiolase (*phaA*), NADPH-dependent acetoacetyl-CoA reductase (*phaB*), and PHA synthase (*phaC*) have been involved in pathway I for the synthesis of PHA. The first enzymatic reaction was catalyzed by β-ketoacyl CoA thiolase (*phbA*) that condensed two molecules of acetyl-coenzyme A (acetyl-CoA) into acetoacetyl-CoA. In the second reaction, the NADPH-dependent (R)-specific acetoacetyl-CoA dehydrogenase/reductase (*phbB*) enzyme reduces the acetoacetyl-CoA molecule into (R)-3-hydroxybutyryl-CoA. Lastly, the enzyme PHA polymerase/synthase (*phbC*) polymerizes the (R)-3-hydroxybutyryl-CoA monomers using PHB precursor into PHB polymer (Muhammadi et al. 2015). The representative of this pathway is *Ralstonia eutropha*. A related pathway involving the degradation of PHA catalyzed by acetoacetyl-CoA synthase, 3-hydroxybutyrate dehydrogenase, PHA depolymerase, and dimer hydrolase that helps to regulate PHA synthesis and its degradation. Bacterial strains of *R. eutropha*, *P. oleovorans*, *P. stutzeri*, and *Aeromonas hydrophila* are the well-known examples of this associated pathway (Sudesh et al. 2000). Recently, Reddy et al. (2020) reported that fatty acids such as acetic acid, butyric acid, propionic acid, and caproic acid were utilized as carbon substrates by *Bacillus* sp. CYR1 for the synthesis of P(3HB-*co*-3 HV) having a higher portion of HB content. Besides, *phaA*, *phaB*, *phaC*, and *phaJ* have been reported to involve during the synthesis of P (3HB-*co*-3 HV) using the PCR-sequencing method (Reddy et al. 2020).

In another study, *phcC* genes and the PHA-associated gene clusters have been examined in four Antarctic isolates, viz., *P.* spp. UMAB-08, *P.* spp. UMAB-40, *Janthinobacterium* spp. UMAB-56 and *Janthinobacterium* spp. UMAB-60 using whole-genome sequence study. *P.* strains were found to comprise Class 1 and Class 2 PHA synthase gene clusters, which are mostly involved in the biosynthesis of SCL-PHA and MCL-PHA respectively. In contrast, the *Janthinobacterium* strains contain only an uncharacterized putative *phbC* and Class I *phbC* genes. On further investigation via multiple sequence alignment, the detected genes of uncharacterized putative *phbC* were found to contain residues of highly conserved amino acid and revealed catalytic triad of *phbC* which was quite distinct from normal Class I, II, III, and IV *phaC*. The findings of in vitro *phaC* enzymatic assay and PHA biosynthesis revealed that this uncharacterized putative *phaC* from *Janthinobacterium* sp. UMAB-60 is active. This report added up new knowledge to the *phaC* database of Antarctic bacterial strains isolated from such geographically isolated and extreme environments with the other normal non-Antarctic PHA-producing microorganisms (Tan et al. 2020).

Table 1.1 Genes and enzymes involved in the synthesis of polyhydroxyalkanoates (PHA) and its copolymers

PHA and its copolymers	Genes and enzymes involved	Carbon sources	Microorganisms	References
Poly (lactate-*co*-3-hydroxybutyrate-*co*-3-hydroxyalkanoate) (P(LA-*co*-3HB-*co*-3HA) with 19.7 mol% LA (C_3), 74.9 mol% 3HB (C_4), and 5.4 mol% MCL-3HA units of C_8 and C_{10}	β-Ketothiolase (*phaA*), NADPH-dependent, acetoacetyl-CoA reductase (*phaB*), propionyl-CoA transferase (*PCT*), D-lactate dehydrogenase (*D-LDH*), (R)-3-hydroxyacyl-ACP thioesterase (*phaG*), (R)-3-hydroxyacyl (3HA)-CoA ligase and modified PHA synthase (*phaC1* (*STQK*))	Glucose	*Recombinant Escherichia coli LS5218*	Goto et al. (2019)
Poly (lactate-*co*-3-hydroxyalkanoate) (P(92.0% LA-*co*-3HA)) with C_3, C_8, C_{10}, and C_{12} monomer units	*PCT*, *D-LDH*, *phaG*, (R)-3-hydroxyacyl (3HA)-CoA ligase and *phaC1*(*STQK*)	Glucose	*Recombinant E. coli CAG18497 strain*	Goto et al. (2019)
Poly ((*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate) (PHBHHx)	*phaB*, NADH-3-hydroxyacyl-CoA dehydrogenase, crotonase ((*S*)-specific enoyl-CoA hydratase)	Soybean oil	Mutant *Ralstonia eutropha H16_A3307*	Segawa et al. (2019)
PHBHHx	(R)-specific enoyl coenzyme-A hydratase (*phaJ*) and PHA synthetase (*phaC2*) with deletion of acetoacetyl Co-A reductases (*phaB1*, *phaB2*, and *phaB3*)	Coffee waste oil	*Ralstonia eutropha Re2133*	Bhatia et al. (2018)
PHBHHx	(*R*)-specific enoyl-CoA hydratase (phaJ_{Ac})	Vegetable oil soybean	*Cupriavidus necator* $\mathit{H16C}_{Ac}$	Mifune et al. (2010)
Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) (P(3HB-*co*-3HV-*co*-3HHx)) terpolymer	Poly (3-hydroxyalkanoate) polymerase or PHA synthase (*phaC*)	Crude palm kernel oil and 3HV precursors	*Mutant Cupriavidus necator*	Bhubalan et al. (2010)

Single chain length-PHA (SCL-PHA) and medium chain length PHA (MCL-PHA)	*pha* Synthase Class 1 (*phaC1*) and *pha* synthase Class 2 (*phaC2*)	Glucose	*Antarctic Pseudomonas* spp. *UMAB-08* and *UMAB-40*	Tan et al. (2020)
	phaC1 and an uncharacterized putative PHA synthase genes	Glucose	*Antarctic Janthinobacterium* spp. *UMAB-56* and *UMAB-60*	
Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3 HV))	*phaA*, *phaB*, *phaC*, and (*R*)-Enoyl-CoA hydratase/enoyl-CoA hydratase I (*phaJ*)	Fatty acids (acetic: propionic acid, butyric acid, caproic acid)	*Bacillus* sp. *CYR1*	Reddy et al. (2020)
P(3HB-*co*-3 HV)	*phaC1*, *phaC2*, *phaZ* (PHA depolymerase), and *phaJ4*	Lauric acid	*Pseudomonas putida KT2440*	Wang and Nomura (2010)
Poly (3-hydroxydecanoate-*co*-3-hydroxydodecanoate) (P(3HD-*co*-3HDD)), with 90.01 mol% 3HD and 58.60 mol% 3HDD	3-Ketoacyl-CoA thiolase (*FadA*), *3*-hydroxyacyl-CoA dehydrogenase (*FadB*), Malonyl-CoA-ACP transacylase (*FadD*), *phaC* and R-enoyl-CoA hydratase (*phaJ*)	Sodium octanoate and sodium decanoate	*Mutant Pseudomonas mendocina NKU-Δβ5*	Zhao et al. (2020)
P(3HD-*co*-3HDD) with 94.71 mol% 3HD and 68.67 mol% 3HDD	*FadA*, *FadB*, *FadD*, *phaC* and *phaJ*	Dodecanoic acid	*Mutant Pseudomonas mendocina NKU-Δβ1*	Zhao et al. (2020)
Polyhydroxybutyrate (PHB)	*phaC1*, Vitreoscilla hemoglobin (*vgb*) and *phaJ5*	Fructose under oxygen limiting condition	Metabolically engineered *Cupriavidus necator H16*	Tang et al. (2020)
Polyhydroxyalkanoate (PHA)	*6-Phosphogluconate dehydrogenase* (*gnd*)	Carbohydrates and plant oils	*Pseudomonas* sp. *LFM046*	Rezende et al. (2020)
Poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P3HB4HB)	4-hydroxybutyrate-CoA:CoA transferase (*OrfZ*), succinic semi aldehyde dehydrogenase (4hbd-*SucD-ogdA*), *orfZ*-encoding enzyme was replaced by γ-butyrolactone (GBL), high-resolution control of gene expressions (HRCGE), fluorescence reporter (*sgfp*)	Glucose	*Recombinant Halomonas bluephagenesis TD68-194*	Ye et al. (2020)

Moreover, microorganisms were reported to implement Pathway II via fatty acid β-oxidation, where acyl-CoA entered into the PHA production process to synthesize mostly MCL-PHAs from fatty acids. The enzymes which are mainly involved in this pathway were found to be 3-ketoacyl-CoA reductase (*phaB*), epimerase, acyl-CoA oxidase (putative), (*R*)-enoyl-CoA hydratase (*phaJ*), and enoyl-CoA hydratase I (putative). These enzymes facilitated the delivery of the 3-HA-CoA PHA precursor for PHA synthesis. In another study, bacterial strains such as *A. hydrophila*, *P. putida*, and *P. aeruginosa* were reported to follow pathway II for the production of poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx), which belongs to MCL-PHA-type polymer (Chen 2010).

Furthermore, pathway VIII was also investigated and the enzymes involved in this Pathway were mainly malonyl-CoA-ACP transacylase (*FabD*) and 3-hydroxyacyl-ACP-CoA transferase (*PhaG*). *FabD* and *PhaG* genes were found to aid 3-hydroxyacyl-ACP for initiating the synthesis of PHA monomer 3-hydroxyacyl-CoA under the accomplishment of the *phaC* gene (Taguchi et al. 1999; Sudesh et al. 2000; Zheng et al. 2005). A mutant *C. necator* PHB4 transformant harboring the *phaC* gene of *Chromobacterium* sp. USM2 bearing prominent attraction towards 3HV monomer was investigated for enhancing the production of P(3HB-*co*-3HV-*co*-3HHx) terpolymer with 3HV monomer units ranging from 2 to 91 mol% using mixtures of crude palm kernel oil and 3HV precursors as carbon sources (Bhubalan et al. 2010). In another investigation, the (*S*)-(+)-3-hydroxybutyryl-CoA was found to be oxidized by *phaB* gene and the mechanism was named Pathway IV. This pathway was investigated in *Rhizobium* (*Cicer*) sp., where NADPH was oxidized to NADP+, leading to the better sourcing of reductant to nitrogenase and also help in the synthesis of PHB by reducing acetoacetyl-CoA (Chohan and Copeland 1998). In a recent study, the heterologous expression of 6-phosphogluconate dehydrogenase (*gnd* genes) from *P. putida* KT2440 (NAD^+ dependent) and *Escherichia coli* MG1655 ($NADP^+$ dependent) was reconstructed in *P. putida* LFM046 to trigger a delay on cell growth and a reduction in PHA yield, respectively. The findings indicated that the modification in the metabolic pathway (cyclic Entner-Doudoroff or pentose phosphate pathway) could be an interesting strategy for *P. putida* and other bacteria to meet divergent cell growth and PHA production simultaneously (Rezende et al. 2020).

In another study, PHA containing 4-hydroxybutyrate monomer units were found to be synthesized using Pathway V. The key enzymes associated with this pathway were mainly succinic semi-aldehyde dehydrogenase (*SucD*), 4-hydroxybutyrate dehydrogenase (*4hbD*), and 4-hydroxybutyrate-CoA: CoA transferase (*OrfZ*), which help in producing 4-hydroxybutyryl-CoA. *Clostridium kluyveri showed* Pathway V for PHA synthesis (Valentin and Dennis 1997). In another study, the enzymes which were involved in Pathway VI were found to be hydroxyacyl-CoA synthase and putative lactonase which convert 4,5-alkanolactone into 4,5-hydroxyacyl-CoA for the production of PHA (Muhammadi et al. 2015; Valentin and Steinbüchel 1995). In addition, *A. hydrophila* 4AK4 was found to be a representative of Pathway VII, which was associated with the enzyme putative alcohol dehydrogenase to produce PHA. Besides, Pathway VII was also investigated for the synthesis of mostly PHA containing 4HB monomer units. This pathway produces poly (4-hydroxybutyrate) (P4HB) by oxidizing 1,4-butanediol into 4-HB. Further 4-HB was oxidized into 4-hydroxybutyryl-CoA for the production of P4HB (Xie and Chen 2008). Furthermore, Pathway VIII was investigated for the synthesis of PHA containing 6-hydroxyhexanoate monomer units (PHHx). This pathway was associated with eight enzymes, viz., cyclohexanol dehydrogenase (ChnA), cyclohexanone monooxygenases (ChnB), caprolactone hydrolase (ChnC), 6-hydroxyhexanoate dehydrogenase (ChnD), 6-oxohexanoate dehydrogenase (ChnE), semi aldehyde dehydrogenase, 6-hydroxyhexanoate dehydrogenase, and putative and hydroxyacyl-CoA synthase enzymes. These enzymes are responsible for the transformation of 6-HHx into PHHx polymer (Chen 2010). In a report, variation in the gene expression levels involved in the glycerol metabolism, TCA cycle, Entner-Doudoroff pathway, β-oxidation, and PHA synthesis by *P. putida* KT2440 using glucose, glycerol, citrate, and lauric acid as a sole carbon source were detected using quantitative real-time PCR. It was observed that gene expression associated with glycerol metabolism was improved with the deletion of *glpR* gene. The Entner-Doudoroff pathway was found to involve putative enzymes which helps in growing the cells using glucose and other carbon sources. The findings showed no significant differences in gene expression levels. On the other hand, from the TCA cycle, genes exhibited superior expression levels in the cells grown on lauric acid and citrate. When cells were grown on lauric acid, a higher expression level was observed in the fatty oxidation complex alpha subunit (*fadB*) and the long-chain fatty acid transporter gene involved for β-oxidation. For the synthesis of PHA using lauric acid, genes encoding enzymes *phaZ*, *phaC1*, *phaJ4*, and *phaC2* were found to exhibit better expression levels (Wang and Nomura 2010). This investigation had identified genes entangled in the metabolism of various carbon sources as well as helps in understanding the PHA biosynthesis more extensively (Table 1.1).

1.4 Chemical Structure and Classification of Microbial Plastic

The microbial plastics (PHAs) are a complex class of mostly linear or aliphatic, head-to-tail, and optically active polyoxoesters which are comprised of (R)-3-hydroxy fatty acid monomers, as shown in Fig. 1.2 (Anjum et al. 2016). In these polyesters, the carboxyl (-COOH-) group of one monomer forms an ester (-CO-) bond with the hydroxyl (-OH-) group of the neighboring monomer (Muhammadi et al. 2015). Due to the stereospecificity of PHA biosynthetic enzymes, the -OH-substituted carbon (C) atom present in PHAs is of the R-configuration (Fig. 1.2). Thus, the chemical synthesis of PHA is difficult due to the presence of chiral (R) center in its backbone. At the same C-3/β position, an alkyl group which can vary from methyl (-CH_3-) to tridecyl is positioned (Fig. 1.2) (Koller 2018). However, this alkyl side chain is not necessarily saturated. Some unsaturated, halogenated, aromatic, epoxidized, and branched monomers have also been reported. The bulk of PHAs are mainly composed of R(-)-3-hydroxyalkanoic (HA) acid monomers ranging from C_3 to C_{14} carbon atoms with a variety of unsaturated or saturated and

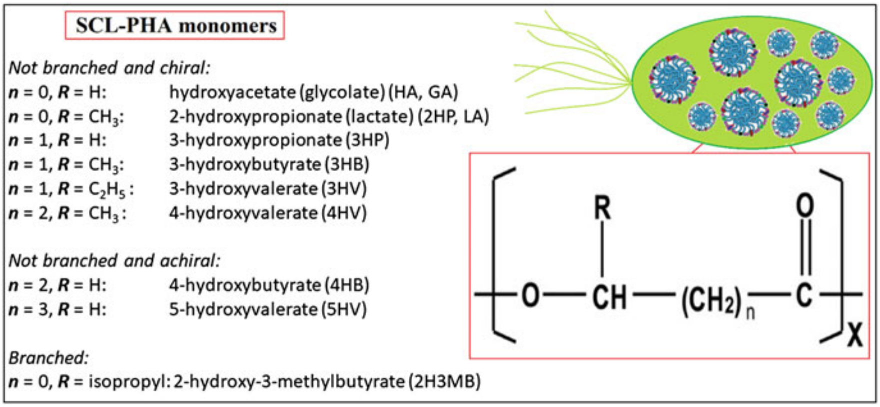

Fig. 1.2 Structure of polyhydroxyalkanoate (PHA) and short-chain-length PHA monomers

branched or straight chain containing aromatic or aliphatic side groups (Muhammadi et al. 2015; Anjum et al. 2016; Koller 2018). So far, more than 150 dissimilar hydroxyalkanoic (HA) acids have been identified that exist as monomer units of PHAs. The variation in the number is remains increasing with the introduction of novel PHA copolymers via physiochemical variation or by the improvement of genetically engineered microorganisms into improved PHA having specific functional groups and better material properties than the natural PHA (Muhammadi et al. 2015). The molecular weight of the PHA was found to be in the range of 2×10^5 to 3×10^6 Da. The molecular weight mostly depends on the number of carbon atoms constituting the monomer units and the type of microorganism and its growing parameters (Koller 2018; Bugnicourt et al. 2014).

PHAs are categorized into four groups, viz., short-chain-length PHAs (SCL-PHAs), medium-chain-length PHAs (MCL-PHAs), long-chain-length PHAs (LCL-PHAs), and random copolymer PHAs, based on the number of carbon atoms existing in the monomer units (Muhammadi et al. 2015). The first group SCL-PHAs, comprising three to five (C_3–C_5) carbon atoms in the monomer unit such as 3HB, 4HB, 3HV, 4-hydroxyvalerate (4HV), 3-hydroxypropionate (3HP), 5-hydroxyvalerate (5HV), and its copolymer P(3HB-*co*-3HV) as shown in Fig. 1.2. The second group MCL-PHAs, comprising 6–14 (C_6–C_{14}) carbon atoms in the monomer units, such as 3HHx, 3HO, 3-hydroxynonanote (3HN), 3-hydroxydecanoate (3HD), 3-hydroxyheptanoate (3HHp), and 3-hydroxydodecanoate (3HDD) as shown in Fig. 1.3 (Koller 2018). The SCL-PHAs are highly crystalline, stiff, and brittle, whereas MCL-PHAs have lower crystallinity and good elasticity. The MCL-PHAs and their copolymers have diverse physiochemical, thermal, and mechanical properties than SCL-PHAs. The MCL-PHAs exist as amorphous liquids or semi-crystalline elastomers due to their low glass transition temperature (T_g). The T_g increases with increasing the average polymer chain length, MCL-PHAs from elastomeric polymers at specific side-chain lengths. Highly viscous and sticky polymers are formed on a further increase in the

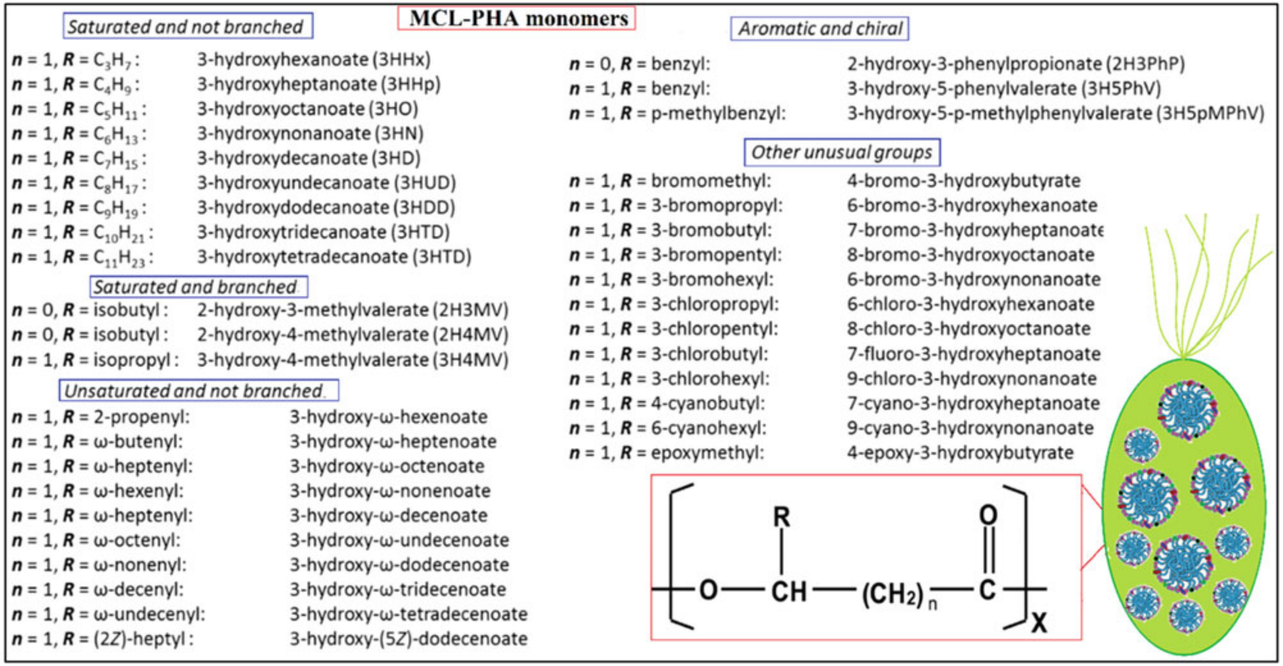

Fig. 1.3 Medium-chain-length PHA monomers

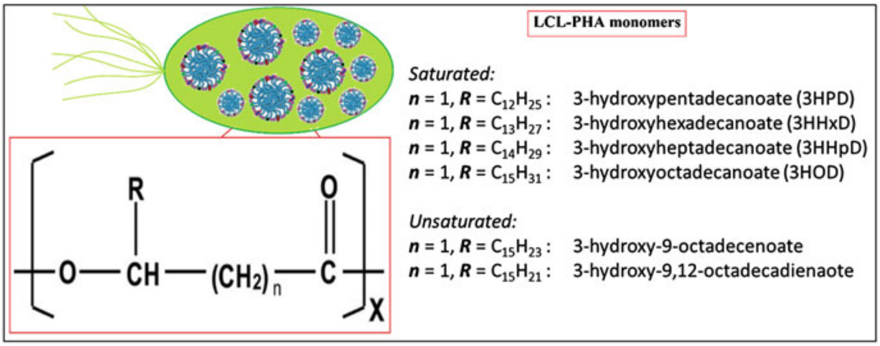

Fig. 1.4 Long-chain-length PHA monomers

side chain length of the PHA (Kai and Loh 2014). MCL-PHAs function as elastomeric materials within a narrow temperature range because of their low melting temperature (T_m). At temperatures above or near to its T_m, the MCL-PHA loses its crystallinity and becomes adhesive. Hence, it is not easy to use MCL-PHAs as flexible biomaterials (Koller 2018). PHAs with required physical properties can be formulated and customized for specific applications through structural modification (Sathiyanarayanan et al. 2017). The third group LCL-PHAs, containing monomer units with more than 14 carbon ($\geq C_{14}$) atoms such as 3-hydroxyhexadecanoate (3HHxD), 3-hydroxypentadecanoate (3HPD), 3-hydroxyheptadecanoate (3HHpD), 3-hydroxy-9-octadecenoate, 3-hydroxyoctadecanoate (3HOD), and 3-hydroxy-9,12-octadecadienaote is shown in Fig. 1.4 (Koller 2018; Roja et al. 2019).

The fourth group is the random PHA copolymer, which is the random copolymerization between SCL-PHAs and MCL-PHAs or between SCL-PHAs and LCL-PHAs or any random PHAs. This separate PHA group has been categorized because of the substrate specificity of the PHA synthesis, which can accept only HAs or 3HAs of a precise range of carbon chain. In the reported *Alcaligenes eutrophus* strain, *phaC* gene can only polymerize 3HAs of SCL-PHAs. However, in the case of *P. oleovorans*, the *phaC* gene can only polymerize 3HAs of MCL-PHAs. In SCL-PHAs, the monomers cannot be oxidized at the C_3 position, whereas in the case of MCL-PHAs, the monomers can be oxidized at the C_3 position except in a few exceptional cases (Muhammadi et al. 2015). There are investigations, where MCL-PHAs have been polymerized with various functional groups, viz., halogens, branched alkyls, cyano, olefins, and aromatics, to improve their material characteristics. It the versatility of PHA structure, which helps in designing and developing preferable novel PHA copolymers having enhanced physiochemical and thermo-mechanical properties (Koller 2020; Muhammadi et al. 2015).

1.5 Microorganisms Producing PHA and Its Co-polymers

Wide varieties of microorganisms such as recombinant and wild strains of *Wautersia eutropha*, *Ralstonia eutropha* (Bhatia et al. 2019a, b), *Bacillus* sp. (Bhatia et al. 2018; Ramadas et al. 2009), *Azotobacter* sp. (Urtuvia et al. 2020), *Haloferax* sp. (Han et al. 2012), *Aeromonas* sp. (Chien and Ho 2008), *Halomonas* sp. (Kulkarni et al. 2015; Thomas et al. 2019), *Thermus thermophilus* (Pantazaki et al. 2003), *Methylobacterium* sp. (Yezza et al. 2006; Amaro et al. 2019), *Chromobacterium* sp. (Kimura et al. 2002; Chek et al. 2017), *Sacchrophagus degradans* (Munoz and Riley 2008; Mudenur et al. 2019), *E. coli* (Amaro et al. 2019), *Cupriavidus* sp. (Benesova et al. 2017) and *Pseudomonas* sp. (Sathiyanarayanan et al. 2017; Rizzo et al. 2019) have been reported for the commercial production of PHA and its copolymers. Most of the prokaryotic microorganisms, including photosynthetic bacteria and halophiles, are reported as a good producer of PHA. Sunlight is the source of energy to produce PHB by cyanobacteria, which resulted in the reduction of CO_2 (a significant greenhouse gas) (Troschl et al. 2017). Cyanobacteria possess PHA storage capacity and could produce PHA by oxygenic photosynthesis (Troschl et al. 2017). Under nitrogen and phosphate limiting conditions, *Synehcystis* sp. or *Synechocystis* sp. could accumulate PHB (Mudenur et al. 2019). Microorganisms which are capable of producing PHAs can be categorized into two main groups on the basis of growth parameters needed for the synthesis of PHA.

The first group of microorganisms requires limited essential nutrients such as phosphorus, nitrogen, magnesium or sulfur for PHA synthesis and a rich carbon substrate (Muhammadi et al. 2015). Some of the examples *Protomonas extorquens*, *Ralstonia eutropha*, and *P. oleovorans*. *Ralstonia eutropha* produce SCL-PHAs and are included in this group (Ciesielski et al. 2014; Muhammadi et al. 2015). Fatty acids or hydrocarbons were fermented by most of the *Pseudomonads* spp. like *P. oleovorans* using 3-hydroxyacyl-CoA intermediates of the β-oxidation pathway to synthesis MCL-PHAs. There are reports where, *Pseudomonas* strain GP4BH1 has synthesized MCL-PHAs comprising 3HB and 3HO monomer units using octanoate and MCL-PHAs containing 3HB, 3HO, and 3HD monomers using gluconate as carbon sources (Steinbüchel and Wiese 1992). In another report, PHB homopolymer blended with a random copolymer poly (3-hydroxybutyrate-*co*-3-hydroxyalkanoates) [P(3HB-*co*-3HA)] containing C_4–C_{12} HA monomer units was synthesized by *Pseudomonas* sp. strain 61-3 using alkanoic acids and sugars (Kato et al. 1996; Abe et al. 1994; Muhammadi et al. 2015). The hydrocarbon degraders are also reported as good producer of PHA. The sites contaminated with oil rich in carbon and poor in nitrogen content create a favorable condition for microbial cells to produce PHA (Mudenur et al. 2019). Many bacterial strains of genera *Ralstonia*, *Caulobacter*, *Yokenella*, *Acinetobacter*, *Pseudomonas*, *Brochothrix*, *Sphingobacterium*, and *Burkholderia* are included in this category (Pan et al. 2012; Sathiyanarayanan et al. 2017). Halophiles require salts to grow and Archaea residing in the oceans, salt lakes, and hot springs have been reported to synthesize PHAs (Mudenur et al. 2019). The marine habitats are abundant in organic matter but habitually limited in nitrogen, oxygen, and phosphorus that create a favorable environment for the marine microorganisms to produce PHA (Thatoi et al. 2013). There are reports where highly halophilic bacteria under nutrient-deficiency conditions could accumulate polyhydroxybutyrate (PHB) (Shrivastav et al. 2011). There are reports, where *Haloferax mediterranei* and *H. boliviensis* were investigated to produce 65% and 56% of PHA of its total cell dry weight (CDW) using glucose and starch hydrolysate respectively (Chen et al. 2006). Oxygen limitations can enhance the production of PHAs (Quillaguaman et al. 2005). Several other sources such as anaerobic or aerobic system, activated sludge, and aerobic dynamic feeding system have been reported for PHA production.

The second group of microorganisms does not involve nutrient limitation for PHA production, in this case the PHA polyester is accumulated during the growth phase. Some of the producers are recombinant *Escherichia coli*, a mutant *Azotobacter vinelandii*, and *Alcaligenes latus*. These features are important to be reflected during the PHA synthesis (Muhammadi et al. 2015). Some of the antibiotic-producing aerobic gram-positive bacterium *Streptomyces* is also investigated for PHA production with some vital metabolites (Valappil et al. 2007). *Alcaligenes eutrophus* was reported to produce copolymers of PHA such as P(3HB-*co*-3HV) and P(3HB-*co*-4HB) (Mudenur et al. 2019). Some of the bacterial species of *Rhodospirillum rubrum*, *Rhodococcus* sp., and *Rhodocyclus gelatinosus* were investigated to produce terpolymer of PHA containing 3HA monomers units of C_4, C_5, and C_6 using hexanoate as major carbon substrate (Dalal and Lal 2019). Besides, a random copolymer of HB and 3-hydroxyhexanoate (HH) was investigated in *Aeromonas caviae* (Wang and Chen 2017). In addition, LCL-PHAs were also investigated in Fluorescent *pseudomonads* spp. (Singh et al. 2018). Many *Pseudomonas* strains (*P. fluorescens*) were reported to synthesize a P(3HB-*co*-3HA) copolymer comprising HA monomer units of C_4–C_{12} from hydroxybutyric acid (HB) and 1,3-butanediol (Cho et al. 2020). A report says that *Thiocapsa pfennigii* was capable to synthesize only a PHB homopolymer using different carbon sources. However, a genetically engineered *P. putida* strain harboring the *phaC* genes of *T. pfennigii* synthesized a terpolymer of PHA, P(HB-HHx-HO) using octanoate as a carbon source (Halevas and Pantazaki 2018; Dalal and Lal 2019). Mysteriously, the existence of PHA is not restricted to the only accumulated intracellular granules. PHB with poor molecular weight ($Mw < 14{,}000$ Da) was also found in *B. subtilis*, *A. vinelandii*, and *Streptomyces lividans* in association with calcium ions and polyphosphate (Dalal and Lal 2019).

Furthermore, there are reports on PHA synthesis by mutant eukaryotic yeast species such as *Saccharomyces cerevisiae* (Sandström et al. 2015; Zhang et al. 2006; Poirier et al. 2001), *Rhodotorula minuta* strain RY4 (Desuoky et al. 2007), *Yarrowia lipolytica* (Gao et al. 2015; Haddouche et al. 2011), and *Pichia pastoris* (Poirier et al. 2002). Recently, indigenous or wild yeast species, viz., *Wickerhamomyces anomalus* VIT-NN01 (Ojha and Das 2018, 2017) and *Candida tropicalis* BPU1 (Prakasan et al. 2016) have also been investigated as potential producers of PHA using cheap carbon sources. A recent report showed 79% of PHBV production using banana peels and chicken feather hydrolysate from marine yeast *Pichia kudriavzevii* VIT-NN02 isolated from seaweeds (Ojha and Das 2020a).

These studies showed the active role of non-halophilic and halophilic yeasts in the accumulation of PHA using cheap raw materials as carbon substrates. Halophilic yeasts would be developed as an effective producer of PHAs due to their unique flexibility formed while persisting under such natural habitat (rhizosphere, mangrove, seaweeds, estuarine sediment) (Ojha and Das 2020a). There is a necessity to discover more eukaryotic wild-type yeast strains for the production of PHA and the mechanism behind it.

1.6 Major Drawbacks of Microbial Bioplastic Production

Currently, only 1% of the plastics produced annually are bioplastics (Lettner et al. 2017; Sabapathy et al. 2020). Companies such as Biocycle PHB Industrial SA (Serrano, SP, Brazil), Metabolix® (Woburn, MA, USA), Tianjin Green Bioscience Co., Ltd. (Tianjin, China), and Procter & Gamble Co., Ltd. (Cincinnati, OH, USA) are the commercial producer of PHAs. Few PHAs and their copolymers such as PHB, poly-4-hydroxybutyrate (P4HB), their copolymer P(3HB-*co*-4HB), copolymers of 3HB and 3HV or P3(HB-*co*-HV), and copolymers of 3HB and 3HHx or P3(HB-*co*-HHX) are being produced in a large scale with limited market success (Możejko-Ciesielska and Kiewisz 2016).

The major cause of the limited success of PHA is the high production costs that are almost three times more than the conventional synthetic plastics polyethene (PE), polypropylene (PP), and polylactic acid (PLA) (Singh et al. 2019). The high production cost is contributed to the high substrate and high processing costs (Kourmentza et al. 2017; Lettner et al. 2017). Moreover, low PHA productivity is also a reason. Strategies such as the utilization of waste carbon sources along with strain improvements have been implemented for the improvement in PHA production.

1.7 Sustainable and Cost-Free Substrates for Microbial Bioplastic Production

A considerable amount of agro-industrial wastes such as sugar cane bagasse, beet molasses, teff straw, wheat straw, corn cob, animal wastes, industrial wastes (leather effluents, chicken feathers), vegetable-fruit wastes (banana, mango, pineapple, orange peels), used vegetable oils (palm, jatropha, sesame oils), food, dairy (Bhatia et al. 2019a, b), and plastic wastes are being disposed of annually and increasing environmental pollution (Getachew and Woldesenbet 2016). These biowastes are promising sources of starch, lignin, cellulose, hemicellulose, hydrocarbons, fatty acids, proteins, and lipids (Kumar et al. 2019), which could be efficiently utilized for viable production of PHA (Ramadas et al. 2009). Various substrates such as sucrose, triacylglycerols, and hydrocarbons were derived from agricultural, domestic, dairy, food, animal, and plastic recycling by-products for the cost-effective production of PHA as shown in Figs. 1.5 and 1.6. The substrates have been classified into three

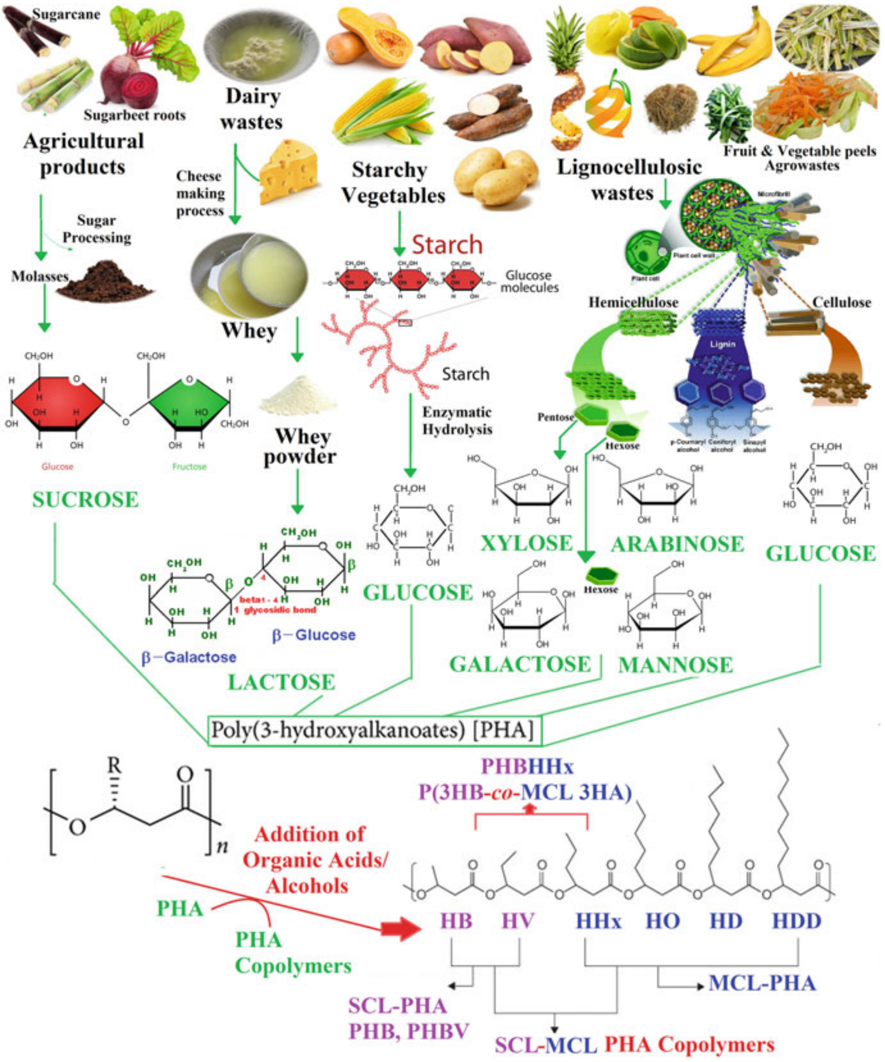

Fig. 1.5 Conversion of inexpensive dairy, agricultural, and lignocellulosic biowastes into simple sugars for cost-effective production of PHA and its copolymers

categories, viz., monosaccharides or simple sugars, triacylglycerol or fatty acids, and hydrocarbons (e.g. crude glycerol, methane). Simple sugars are mostly utilized by PHA-producing microorganisms, whereas triacylglycerol and hydrocarbons have only been utilized by few microorganisms (mostly *Pseudomonas* species). The conversion of different waste carbon sources to PHAs can be referred to as the catalytic transformation of substrates in a chemical process. The bacterial cells act as biochemical catalysts in the biosynthesis of PHAs while carrying out the metabolic

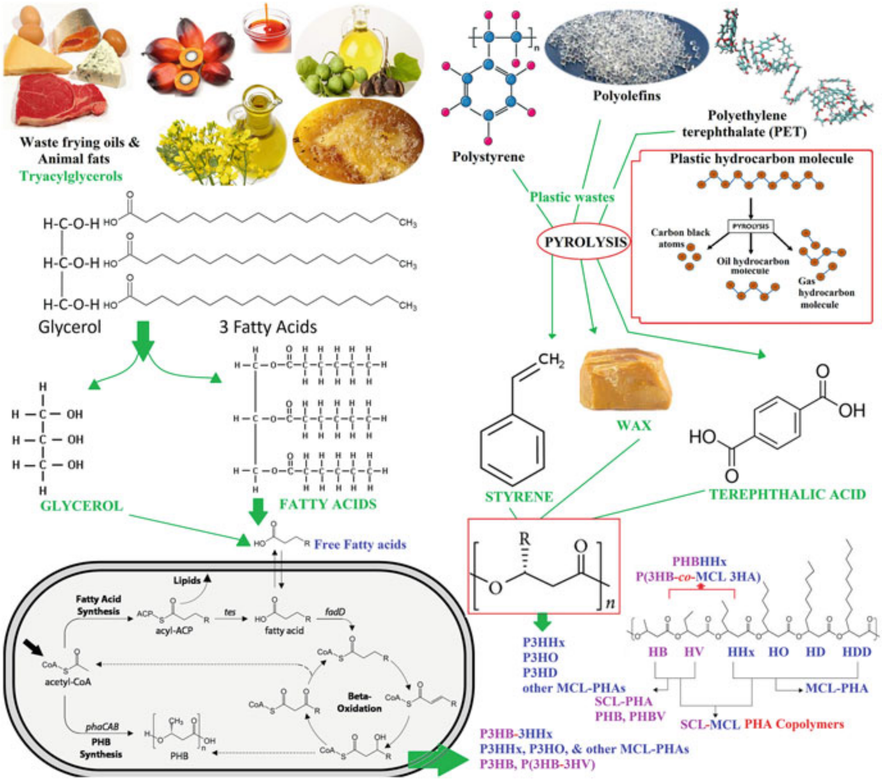

Fig. 1.6 Conversion of waste frying oils, animal fats, and plastic wastes into their respective simpler form, i.e., glycerol, fatty acids, and pyrolysis hydrocarbon products for the inexpensive and sustainable production of PHA and its copolymers

reactions. Chemical catalysts can bind to a specific substrate, and the specific substrate can be converted into different products by varying catalysts (Figs. 1.5 and 1.6). A similar mechanism occurs for PHA-producing microorganisms due to their substrate specificity (Jiang et al. 2016). Different microorganisms can produce PHAs with different compositions using the same substrate. For example, MCL-PHAs, such as poly (3-hydroxylhexanoate) (P3HHx) can be produced by *Pseudomonas* spp. using glucose and other simple sugars, while *Ralstonia eutropha* produces only P3HB using glucose (Jiang et al. 2016). There are reports on the conversion of a large number of waste carbon sources into simpler sugar substrates for fermenting PHA-producing microorganisms to lower the production cost of PHA. Recent updates on the application of waste carbon sources used for the production of PHA and its copolymers using various types of microorganisms have been shown in Table 1.2.

Table 1.2 Sustainable waste carbon sources used for the biosynthesis of PHA and its copolymers using various microorganisms

Cheap carbon sources	Microorganisms	PHA and its copolymers	PHA yield	References
Dairy and food wastes				
Bacterial strains				
Whole/crude whey	*Bacillus megaterium Ti3*	Poly (3-hydroxybutyrate) (P3HB/PHB)	2.20 ± 0.11 g/L and productivity (0.05 g PHA/L/h)	Israni et al. (2020)
Whey permeate and whey retentate	*Hydrogenophaga pseudoflava DSM1023*	PHA	0.6–0.8 g/L	Koller et al. (2019)
Deproteinized dairy wastes	Mixed microbial cultures	Polyhydroxyalkanoate (PHA)	62 ± 4.5% of total dry cell weight (DCW)	Colombo et al. (2019)
Whey	*Caulobacter segnis DSM 29236*	PHB	9.3 g/L of PHB with a yield of 37% of its DCW	Bustamante et al. (2019)
Cheese whey	*Burkholderia sacchari*	PHB	5.14 ± 0.26 wt% 0.06 g/L	de Andrade et al. (2019)
Synthetic and anaerobically digested food waste-derived volatile fatty acids	*Engineered Ralstonia eutropha* Re2133/pCB81	Poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx/P(3HB-*co*-3HHx)) with 18.5% HHx	52% w/w PHA of DCW	Bhatia et al. (2019a, b, c)
Cheese whey permeate	*Bacillus megaterium* NCIM 5472	PHB	8.29 g/L with yield of 75.5% of its total DCW	Das et al. (2018)
Protein-rich cheese whey waste	Mixed microbial cultures (MMCs)	Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate (P(3HB-*co*-3HV)/P3(HB-*co*-HV)/PHBV)	12–14 wt% of total DCW	Oliveira et al. (2018)
Hydrolyzed whey + additives	*Halomonas halophila*	PHB	0.05 g/L/h PHB	Pernicova et al. (2019)
Fermented whey Powder	Mixed microbial cultures (MMCs)	P3(HB-*co*-HV)	0.6 Cmol PHA/Cmol substrate	Gouveia et al. (2017)
Hydrolyzed whey permeate + additives	*Haloferax mediterranei*	P(3HB-*co*-3HV)	0.148 g/L/h	Koller et al. (2015)

Waste frying oils				
Bacterial strains				
Waste sunflower oil combined with digestate chicken manure	*Cupriavidus necator H16*	PHA	75.1 ± 1.4% of DCW	Altun (2019)
Sludge palm oil	*Recombinant Cupriavidus necator Re2058/pCB113.*	P(3HB-*co*-3HHx) P(3HB-*co*-22 mol% 3HHx)	74 wt% of CDW	Thinagaran and Sudesh (2019)
Waste egg yolk	*Cupriavidus necator Re2133/pCB81*	P(3HB-*co*-3HHx)	43.7% of DCW	Hong et al. (2019)
Waste frying oil	*Halomonas hydrothermalis*	P(3HB-*co*-3HV)	50% of the DCW	Pernicova et al. (2019)
Coffee waste oil	Engineered *Ralstonia eutropha Re2133*	PHBHHx with 78 mol% HB and 22 mol % HHx	69% of total DCW	Bhatia et al. (2018)
Used cooking oil	*Burkholderia thailandensis*	PHA	60% of the DCW	Kourmentza et al. (2018)
Vegetable oil (octanoic acid, nonanoic acid, fresh canola oil, waste canola fryer oil, or biodiesel-derived waste free fatty acids)	*Mutant Pseudomonas chlororaphis PA23-63*	MCL-PHA	11.7–32.5% of DCW	Sharma et al. (2017)
Jatropha oil and urea	*Cupriavidus necator H16*	P3HB	87 wt% of DCW	Ng et al. (2010)
Yeast strains				
Cane molasses and waste palm oil	*Wickerhamomyces anomalus* VIT-NN01 (indigenous yeast)	P(3HB-*co*-3HV)	19.50 g/L, 60% of the total DCW	Ojha and Das (2018)

(continued)

Table 1.2 (continued)

Cheap carbon sources	Microorganisms	PHA and its copolymers	PHA yield	References
Lignocellulosic wastes				
Bacterial strains				
Fructose syrup	*Halotolerant Halomonas* sp. *YLGW01*	PHB	95.26 ± 1.78% of the total DCW	Park et al. (2020)
Agave bagasse hydrolysate	*Achromobacter mucicolens*	PHB	31.3 g/100 g of DCW	Clifton-García et al. (2020)
Lignocellulosic feedstock (activated sludge)	*Mixed microbial cultures (MMCs)*	PHBV	637.556 mg/L	Yin et al. (2019)
Plant biomass hydrolysate	*Ralstonia eutropha 5119*	PHA		Bhatia et al. (2019a)
Barley biomass hydrolysate			1.8 g/L	
Miscanthus biomass hydrolysate			2.0 g/L	
Pine biomass hydrolysate			1.7 g/L	
Madhuca indica flower extract	*Ralstonia eutropha*	PHBV	1.44 ± 0.016 g/L with yield of 49.4% of DCW	Kerketta and Vasanth (2019)
Chicken feather hydrolysate with waste frying oil	*Cupriavidus necator*	P(3HB-*co*-3HV)	50% of the total DCW	Benesova et al. (2017)
Xylose-based hydrolysate from eucalyptus extract	Engineered *Escherichia coli*	Poly (lactate-*co*-3-hydroxybutyrate)	5.4 g/L, 62.4% of the total DCW	Takisawa et al. (2017)
		P(LA-*co*-3HB)		
Fruit peel wastes	*Halomonas campisalis118 MCM B-1027*	P3(HB-*co*-HV)		Kulkarni et al. (2015)
Bagasse extract			0.36 g/L	
Orange peel extract			0.33 g/L	
Banana peel extract			0.11 g/L	

Yeast strains				
Banana peels and chicken feather hydrolysate	*Halophilic Pichia kudriavzevii VIT-NN02*	P(3HB-*co*-3HV)	79.68 ± 0.2% of the total DCW	Ojha and Das (2020a, b)
Starch	*Candida tropicalis* BPU1 (wild type)	PHB	39% of total DCW	Prakasan et al. (2016)
Casamino acids, dextrose, oleic acid, tween 80, triolein	*Yarrowia lipolytica* (genetically engineered)	PHB	5% of total DCW	Gao et al. (2015)
Xylose	*Saccharomyces cerevisiae* (genetically modified)	PHA	2% of the total DCW	Sandström et al. (2015)
Plastic recycling wastes and their by-products				
Bacterial strains				
Glycerol	*Stenotrophomonas rhizophila*	(PHB-*co*-HV)	13.7 g/100 g DCW	Clifton-García et al. (2020)
Glycerol	*Antarctic Pseudomonas* sp. MPC6 and *Pseudomonas* sp. *MPC5*	MCL-PHA, poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydecanoate-*co*-3-hydroxydodecanoate) (P(3HB-HHx-HO-HD-HDD))	2.6 g/L, 47.3% of the DCW 1.3 g/L, 34.5% of the DCW	Pacheco et al. (2019)
Glycerol with aquawaste fish oil	*Salinivibrio* sp. *M318*	Poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P(3HB-*co*-4HB)) and P(3HB-*co*-3HV)	51.5 wt% of the DCW	Van Thuoc et al. (2019)
Waste glycerol	*Zobellella taiwanensis Azu-IN1*	P(3HB)	3.73 g/L, 61.7% (w/w) of the total DCW	Abdel-Rahman et al. (2017)

(continued)

Table 1.2 (continued)

Cheap carbon sources	Microorganisms	PHA and its copolymers	PHA yield	References
Glycerol	*Arctic psychrotrophic bacterium Pseudomonas* sp. *PAMC 28620*	MCL-PHA copolymer	52.18 ± 4.12% of DCW	Sathiyanarayanan et al. (2017)
		Poly (3-hydroxyoctanoate-*co*-3-hydroxydecanoate-*co*-3-hydroxydodecanoate-*co*-3-hydroxytetradecanoate) (P(3HO-*co*-3HD-*co*-3HDD-*co*-3HTD))		
Styrene	*Pseudomonas putida* NBUS12	MCL-PHA, Poly (3-hydroxyhexanoate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydecanoate-*co*-3-hydroxydodecanoate-*co*-3-hydroxytetradecanoate) (P3(HHx-HO-HD-HDD)) with 2:42:1257:17:1 mol% monomer units	32.49 ± 2.40% from 1.28 ± 0.10 g/L DCW	Tan et al. (2015)
Waste glycerol	*Zobellella denitrificans MW1*	P(3HB)	87% of total DCW	Ibrahim and Steinbüchel (2010a, b)

1.7.1 Dairy Wastes Used for PHA Production

Whey is the by-product of the dairy (cheese and casein production) industry. Whey permeate is a good source of lactose (approx. 35–50 g/L), a disaccharide comprising galactose and glucose via a glycoside linkage (Fig. 1.5). There are reports where bacterial strains such as *Hydrogenophaga pseudoflava* DSM1023 (Koller et al. 2019), *Sinorhizobium meliloti* (Favaro et al. 2019), *Methylobacterium* sp. ZP24 (Yellore et al. 1999; Amaro et al. 2019), *Thermos thermophilus* HB8 (Jiang et al. 2016), *Caulobacter segnis* DSM 29236 (Bustamante et al. 2019), *Burkholderia sacchari* (de Andrade et al. 2019), and *Bacillus megaterium* (Israni et al. 2020) were investigated to synthesis PHA and its copolymers using whey as its carbon substrate. Some strains like *P. hydrogenovora* could not consume lactose directly. Therefore, whey permeate was enzymatically hydrolyzed into galactose and glucose hydrolysates, which were further utilized for the synthesis of PHA (Koller et al. 2008). However, the productivity and PHA yield of the two procedures are significantly lower than employing the genetically modified strain of *E. coli* harboring *Alcaligenes latus phbC* genes (Choi et al. 1998). Though lactose extracted from whey can be consumed for PHA production, the possibility of whey waste would fulfill only a small portion of the total production of PHA.

1.7.2 Agro-Industrial Wastes Used for PHA Production

Sugar molasses was reported as a sustainable and inexpensive source of sucrose for the effective production of PHA (Ojha and Das 2018). A considerable quantity of gelatinous molasses is yielded as a by-product with rich sucrose content during the production of sucrose from sugar beets or sugarcane. Beet molasses were utilized for the production of PHA using *Azotobacter vinelandii* by fed-batch culture techniques. The total PHA production was found to be 66% of the total cell dry weight (CDW) with a yield of 0.12 g PHA/g of molasses consumed (Page 1992). Molasses has been broadly utilized as a carbon substrate in commercial-scale fermentation due to their low cost and abundance. Still, the possibility of the accessibility of molasses is limited to fulfill the growing demand for the production of PHA.

Agricultural products such as rice, cassava, potato, corn, and sorghum are excellent sources of starch (Gowda and Shivakumar 2014). Starch is a polymer constituent of D-glucose with β-1,4-glycosidic bonds. It is hydrolyzed into maltose and then glucose by enzymatic or acid hydrolysis and becomes the most widely consumed carbon substrate for large-scale production of PHAs (Fig. 1.5). Imperial Chemical Industries (ICI) is the earliest commercial PHB producer using starch-derived glucose by *R. eutropha* (Haas et al. 2008; Jiang et al. 2016). *Alcaligenes latus* (Jiang et al. 2016) and engineered *Escherichia coli* (Bhatia et al. 2015) have also been reported as a commercial producer of P3HB using starch as the starting substrate. However, for the production of PHA copolymers, the ICI technology necessitates a propionic acid as the co-carbon substrate along with the primary carbon source for synthesizing the required copolymer (Jiang et al. 2016). Organic acids and alcohols are served as precursors for the biosynthesis of PHA copolymers. For example, propionic acid is supplemented at the late steps of fermentation to synthesize P(3HB-*co*-3HV) copolymer utilizing glucose as the sole carbon substrate (Jiang et al. 2016; Jung et al. 2019a; Muniyasamy et al. 2019). There are reports where organic acid-rich acidogenic liquid produced from waste sludge anaerobic fermentation was exploited as a possible feedstock for PHA production, which is a cost-effective and environmentally sustainable PHA production system. The maximal PHA content accounted for 60.3% of the CDW with initial fermentation pH 10.0 sludge acidogenic liquid, and the recovered polymer composition was 98.3% PHB and 1.7% polyhydroxyvalerate (PHV) by mass (Liu et al. 2020).

1.7.3 Lignocellulosic Wastes Used for PHA Production

The utilization of lignocellulosic feedstocks has been reported to be the most capable and green method regarding the production of PHA as it completely eliminates the depletion of food crops. The usage of these renewable and green feedstocks could able to develop affordable PHAs production and enable the inexpensive commercialization of waste carbon-based microbial bioplastics production (Al-Battashi et al. 2019). Lignocellulose contains lignin (20–30%), cellulose (40–50%), and hemicelluloses (20–50%), considered the most rich and feasible carbon source. Hemicellulose and cellulose are important sources of consumable sugars; yet, lignin is not degradable because of its dense aromatic structure (Dietrich et al. 2019). Cellulose consists of beta-1,4-glycosidic bonds of D-glucose that is neither digestible by human beings nor soluble in water due to its high molecular weight and crystalline structure. Hemicellulose is an amorphous polysaccharide of various pentose sugars such as xylose, galactose, rhamnose, mannose, and arabinose. Therefore, cellulose and hemicelluloses are separated from the lignin by a process called delignification to grasp high sugar yields for the microorganisms to produce PHA. Steam pretreatment with dilute acids followed by cellulase enzyme pretreatment is mostly processed to degrade these solid biowastes into simpler forms of sugar monomers (Prem Anand et al. 2010). Along with the pentose sugars, the hemicellulose hydrolysates also contain acid-soluble lignin, acetic acid, a slight quantity of hexose, alcohols, dehydrated hexose, and volatile acids. The pure forms of pentose sugars are used for synthesizing PHAs (Bertrand et al. 1990). On comparing the PHA production rate between the pentose sugars (xylose and arabinose) and glucose using *P. pseudoflava*, pentose as substrates had shown much lower productivity than glucose as a substrate (Bertrand et al. 1990; Jiang et al. 2016). Moreover, the lower molecular weight of P3HB using xylose and arabinose during fermentation was observed as compared to glucose. However, the mechanical properties of the PHA biomaterials remain unaffected by variations in molecular weight. *Pseudomonas cepacia* synthesized 48–56% (w/w) of P3HB per biomass with a yield of 0.11 g PHB/g xylose. However, engineered *E. coli* harbored with *phbC* genes of *Ralstonia eutropha* was capable to synthesize 74% PHB per biomass with a yield of 0.226 g PHB/g xylose, which is superior to indigenous strain (Silva et al. 2004; Jiang et al.

2016). Direct use of the hemicellulose hydrolysates as a mixture of sugars has also been studied. Furthermore, the findings showed that low concentrations of hemicellulose hydrolysates could be fermented by *R. eutropha* mostly for cell growth under fed-batch fermentation. Recently, the growth conditions of *R. eutropha* 5119 was optimized to produce PHA using lignocellulosic biomass hydrolysates (LBHs), including Miscanthus biomass hydrolysate (MBH), barley biomass hydrolysate (BBH), and pine biomass hydrolysate (PBH) as effective carbon substrates (Bhatia et al. 2019b).

Glucose can be produced from cellulose via saccharification, which is mainly consumed to yield second-generation ethanol (Koller 2017). A hydroxymethyl furfuran-resistant mutant *E. coli* was reported to produce 58% P3HB of the total CDW of 5.6 g/L using cellulose hydrolysate. Utilization of cellulose and hemicellulose hydrolysate mixture was found to be a favorable tactic to synthesize PHA from lignocellulosic biowastes (Fig. 1.5). *Burkholderia sacchari* yields 0.19 g P3HB/g of the 60% CDW using wheat straw hydrolysates (mainly contained glucose, xylose, and arabinose). In comparison, pentose or hexose sugars produced 0.18 g/g PHB of the 70% CDW (Jiang et al. 2016; Bhatia et al. 2019b). The utilization of glucose as a primary carbon source along with liquefied wood as a low-cost co-substrate is another interesting example to add 3-hydroxyvalerate (3HV) monomer units to produce PHA copolymers. Liquefied woods are cost-effective than commercially available co-carbon substrates, such as propionic or pentanoic acid. In addition, the wood-cellulose-derived levulinic acid has also been investigated as a co-carbon substrate for the production of 3HV containing PHA copolymer (Koller et al. 2015).

There are reports where lignocellulosic, domestic, and agro-industrial wastes such as glycerol, plant biomass hydrolysate, agro-industrial residues, starch hydrolysate, sugar maple hemicellulosic hydrolysate, whey, chicken feather hydrolysate with waste frying oil, jatropha biodiesel, agro-wastes, and sugarcane have been utilized as cheap carbon sources for the PHA production employing bacterial strains such as Arctic psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620 (Sathiyanarayanan et al. 2017), *Ralstonia eutropha* 5119 (Bhatia et al. 2019b), *Halomonas boliviensis* (Quillaguaman et al. 2005), *Burkholderia cepacia* (Pan et al. 2012), *C. necator* (Benesova et al. 2017), *Halomonas campisalis*118 MCM B-1027 (Kulkarni et al. 2015), and recombinant consortia of *Ralstonia eutropha* and *Bacillus subtilis* (Bhatia et al. 2018). Moreover, there are reports on the production of PHA using indigenous yeast species such as *Wickerhamomyces anomalus* VIT-NN01 (Ojha and Das 2018), *Candida tropicalis* BPU1 (Prakasan et al. 2016), and marine *Pichia kudriavzevii* VIT-NN02 (Ojha and Das 2020a), using sugarcane molasses with palm oil, orange peel, and banana peel hydrolysate, respectively, as its primary carbon substrates (Fig. 1.5).

1.7.4 Waste from Frying Oils and Animal Fats for PHA Production

Used frying oils are discarded as industrial waste after numerous uses is another example of an economical and natural source of plant oils for the synthesis of PHA as shown in Fig. 1.6. Waste frying oil comprises triacylglycerol (70%) and a small portion of polymeric or oligomeric monoacyl-glycerol, diacylglycerol, triacylglycerol, aldehydes, ketones, and free fatty acids (Winnacker 2019). There are reports where propanol and waste rapeseed oil were utilized as a co-carbon substrate for producing P(3HB-*co*-3HV) by *R. eutropha*. Also, *Bacillus* sp. CYR1 was reported to utilize the fatty acids for its growth and P(3HB-*co*-3 HV) copolymer production with high hydroxybutyrate content (Reddy et al. 2020).

Waste animal fats from slaughtering and food processing industries have vast potential as a cheap carbon source for the production of PHA (Winnacker 2019). On the other hand, the high melting temperature of waste animal fats can be challenging throughout the fermentation process. *Pseudomonas resinovorans* was reported to synthesize PHA polymer utilizing unhydrolyzed tallow, with 15% PHA content of the total biomass (Pagliano et al. 2017). Moreover, *P. citronellolis* was reported to produce MCL-PHA of 0.036–0.050 g/L and PHA contents of 20.1–26.6 wt% using methanol and ester of the fatty acids obtained from tallow (Pagliano et al. 2017). *Ralstonia eutropha* produced 80% P3HB of the CDW with a 1% 3HV unit using animal fats (Jiang et al. 2016).

Crude glycerol is a by-product obtained during the transesterification of vegetable oils or animal fats. Conversion of the crude glycerol into PHA and its copolymers is a securing way to stabilize the biodiesel production cost and valorization of crude glycerol (Ray et al. 2018). *Ralstonia eutropha* exhibited a maximum of 62% P3HB content of the CDW using glycerol. However, P(3HB) content was found to be 38% of the CDW by *R. eutropha* using crude glycerol (Jiang et al. 2016). Recombinant *E. coli*, *P. corrugate*, and *P. oleovorans* have also been attempted for the production of 3-hydroxybutyric acid and other MCL-hydroxyalkanoic acids containing PHA copolymers using a lower concentration of glycerol 3% (w/v). Because at higher concentrations of glycerol, P3HB content, molecular weight and the specific growth rate of the strains decrease, which could be attributed to low enzymatic efficiency, high osmotic stress, and earlier termination by glycerol (Andreeßen et al. 2010; Volova et al. 2019). *Methylobacterium* sp. is capable of growing on methanol. Therefore, *Methylobacterium organophilum* was also reported to produce P3HB using a low concentration of methanol 0.5% (v/v) under potassium limitation (Zúñiga et al. 2011).

Biorefineries are the developing concept for converting a large amount of lignocellulosic biowastes into biofuels. The biorefinery by-products of waste plant oils, crude glycerol, low-grade biodiesels, and hemicellulose hydrolysates might be proved as feasible green substrates for PHA and its copolymers production (Jiang et al. 2016). However, extensive investigation is required to screen highly efficient strains and to optimize growth parameters to enhance the productivity and yields of PHAs for effective conversion.

1.7.5 Plastics Wastes for PHA Production

Plastics wastes are generally pyrolyzed to generate wax, which is used in ship engines and power plants to generate energy. Pyrolysis is a process where plastic particles are thermally decomposed at a temperature of 450–550 °C exhausting pre-heated nitrogen gas in the fluidized bed into small hydrocarbon molecules (Jiang et al. 2016). Various pyrolysis products (oils and waxes) from different types of plastic wastes (polystyrene, polyolefins, and polyethene terephthalate) have been studied for synthesizing PHA by hydrocarbon-degrading bacterial strains as shown in Fig. 1.6. Many microorganisms are reported to metabolize hydrocarbons containing up to 44 carbon such as long-chain paraffinic, olefinic, 1-alkenes (C_1–C_6), and gaseous *n*-alkanes (Timm and Steinbüchel 1990). However, only a few hydrocarbon-utilizing microorganisms can synthesize PHAs using these hydrocarbons as their primary carbon substrate. *P. oleovorans*, fluorescent *Pseudomonads* and *P. aeruginosa* have been reported to produce MCL-PHAs using octane, *n*-alkanoic acids and hexadecane respectively (Fernández et al. 2005).

Oil produced from pyrolysis of polyethene terephthalate (PET) generates aromatic hydrocarbons (Kourmentza et al. 2017). These aromatic hydrocarbons have been used for the fermentation of PHAs by several *Pseudomonas* strains and *Rhodococcus aetherivorans* IAR1 (Hori et al. 2009). Styrene, polyethene wax, and terephthalic acid have been used to ferment *Pseudomonas* sp. to produce MCL-PHAs (Guzik et al. 2014). Moreover, polyethene wax was used as the carbon source by *R. eutropha* to synthesize PHB with a minor fraction of other MCL-HA units (Johnston et al. 2017). Nevertheless, the mainstream of production process using plastic recycled wastes are still at their initial stages. Wide improvements are required to improve both PHA content and productivity.

1.8 Cost-Effective Microbial Bioplastic Production Involving Extremophiles

Attempts have been made to utilize glucose derived from cellulose for the synthesis of PHAs, but the commercial cost of cellulase preparations remains to hamper the major economic transformation of lignocellulosic biowastes (Jiang et al. 2016). Development of a green-associated bioprocessing method for the single-step conversion of lignocellulose biowastes into PHA polymer using thermophiles excludes the supplementation of enzymes and pretreatment steps which could be cost-effective at lower investment. This may be reflected as a significant development for the bioplastic industry. Thermophilic bacteria retain highly active thermostable ligninolytic, hemicellulolytic and cellulolytic enzymes for capable biomass hydrolysis (David et al. 2018; Bhalla et al. 2013). Attractively, very rare thermophilic strains such as *Geobacillus* sp. AY 946934 (Giedraitytė and Kalėdienė 2015), *Chelatococcus* sp. (Ibrahim et al. 2010, 2016; Ibrahim and Steinbüchel 2010a, b), *Thermus thermophilus* HB8 (Pantazaki et al. 2005, 2003), *Caldimonas manganoxidans* (Hsiao et al. 2016), and *Bacillus shackletonii* K5 (Liu et al. 2014) have been reported to produce PHB. *Synechococcus* MA19 are thermophilic cyanobacterium strain reported to produce PHB photoautotrophically, up to 62% (w/w) of CDW consuming carbon dioxide (CO_2) as key carbon, under nitrogen-limited conditions (Nishioka et al. 2001). P(3HB-*co*-3HV) was reported to synthesis by *Aneurinibacillus* species using glucose as a substrate at 55 °C (Xiao et al. 2015). Moreover, MCL-PHAs were produced by *Pseudomonas* sp., using dodecanoate, octanoate, and acetate as primary carbon sources at 55 °C.

Methane is a hydrocarbon which is easily available in oilfields and during the biological degradation of organic matter. It is a potential substrate for PHA production. A group of type II methylotrophs are known to accumulate P3HB using methane. A yield of 0.55 g PHB g/methane and productivity of 0.031 g PHB were reported using *Methylocystis* sp. (Rostkowski et al. 2013). However, to enhance the PHA production by type II methylotrophs, optimization of growth parameters is yet to be explored.

Many halophilic *Halomonas* sp. have been reported to illustrate the possibility of next-generation industrial biotechnology (NGIB) to reduce PHA production cost and expand PHA diversity underwater and energy-saving conditions (Ling et al. 2018). The wild-type *H. bluephagenesis* are resistant to microbial contamination and found to be a potential producer of PHA including PHB, P34HB, and PHBV (Ye et al. 2018). *Halomonas bluephagenesis* consumes NADH instead of NADPH as a cofactor for the synthesis of PHB. To enhance the PHA production, the NADH/NAD+ ratio was increased under oxygen limitation. This was done by genetic modification of the wild strain, where an electron transport pathway comprising electron transfer flavoprotein subunits α and β encoded by etf operon has been blocked to improve NADH supply. Acetic acid was used as an inexpensive co-carbon substrate along with glucose to reduce and balance the redox state inhibition on pyruvate metabolism which resulted in 94% PHB accumulation and 22% more CDW as compared to the wild-type strain (84%) (Ling et al. 2018). Consequently, a strategy of systematic modulation on the redox potential of mutant *H. bluephagenesis* led to better production of PHA under reduced energy consumption, oxygen limitation, and scale-up complexity.

1.9 Innovative Research on Microbial Bioplastics

PHAs have excellent physicochemical properties which warrant the commercial exploitation of biopolymers in different field of applications, viz., food packaging, biomedical implant materials, drug delivery carriers, automotive, infrastructure and aerospace to military applications (Ojha and Das 2018, 2020b). PHB and poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) are considered the most familiar biomaterial for food packaging among all the classes of biopolymers because of their biocompatibility and biodegradability. Yet, naturally synthesized PHB polymers have restricted applications because of their hydrophobic and brittle nature (Pérez-Arauz et al. 2019). Blending of numerous monomers for the production of 3-HB-based copolymers was developed to enhance the thermal and mechanical properties of the PHB and PHBV. Unfortunately, despite their promising commercial potential, most of the produced PHAs, especially those with a higher monomeric composition of 3-hydroxybutyric acid have been reported to exhibit narrow processing window, hydrophilicity, poor barrier performance, brittleness, severe bacterial infection, poor heat resistance, low thermal, mechanical and structural properties mechanical which are limiting their processing malleability, ductility and restrict their wide applications (Ojha and Das 2020b). The lack of adequate flexibility is the main problem of PHAs which restrict their application as food packaging material.

Nowadays, nanostructured materials revealed various facets for their structural elements/clusters such as nanoparticles/nanoclusters (zero dimensions), nanorods/nanotubes (one dimension), nano-thin films (two dimensions), and nanomaterials (three dimensions) ranging from 1 to 100 nm in size. These nanostructured materials are bearing a superior surface volume ratio and reveal unique physicochemical characteristics, such as strength, magnetism, diffusivity, toxicity, optics, solubility, color, and thermodynamics (Goudarzi and Shahabi-Ghahfarrokhi 2018). Recently, the incorporation of these nanostructured materials into PHA polymers has attracted great research interest due to their versatile properties (Jaiswal et al. 2019). Combining nanostructured materials (>100 nm) with PHA using various fabrication methods leading to the formation of PHA nanocomposites as shown in Fig. 1.7 and Table 1.3.

1.9.1 PHA Nanocomposites

Nanostructured materials have become attractive materials because of their excellent features such as extreme surface area, lesser content condition, and higher strength. The nanomaterials functioned as nucleating agents, increasing the degree of crystallinity and the crystallization temperature of the matrix. It also acts as mass transport barriers and hinders the diffusion of volatiles caused during the decomposition process, resulting in enhanced thermal stability (Pradhan et al. 2015; Ojha and Das 2020b). These nanomaterials are proved to be ideal reinforcing filling agents in biopolymer composites as they possess exceptional mechanical and thermal conductivity with a low coefficient of thermal expansion. Enhanced thermal, mechanical and barrier performances have been achieved upon supplementation of minor amounts of nano-sized fillers into the PHA matrix (Maiti et al. 2007). Besides, the incorporation of bioactive (e.g. antimicrobial, antioxidant, and oxygen-scavenging) agents resulted in the development of efficient antibacterial packaging material which could control the microbial growth and preserve the desirable quality (Basumatary et al. 2018). PHAs can be blended with other polymers (nanofibers) or filling with nano-fillers such as multi-walled carbon nanotubes (MWCNTs), bioactive glass, organically modified montmorillonite (OMMT), nanocrystals, and nanoparticles to formulate PHA nanocomposites for enhancing its thermal, mechanical, and barrier properties (Maiti et al. 2007; Prakalathan et al. 2014; Ojha and Das 2020b).

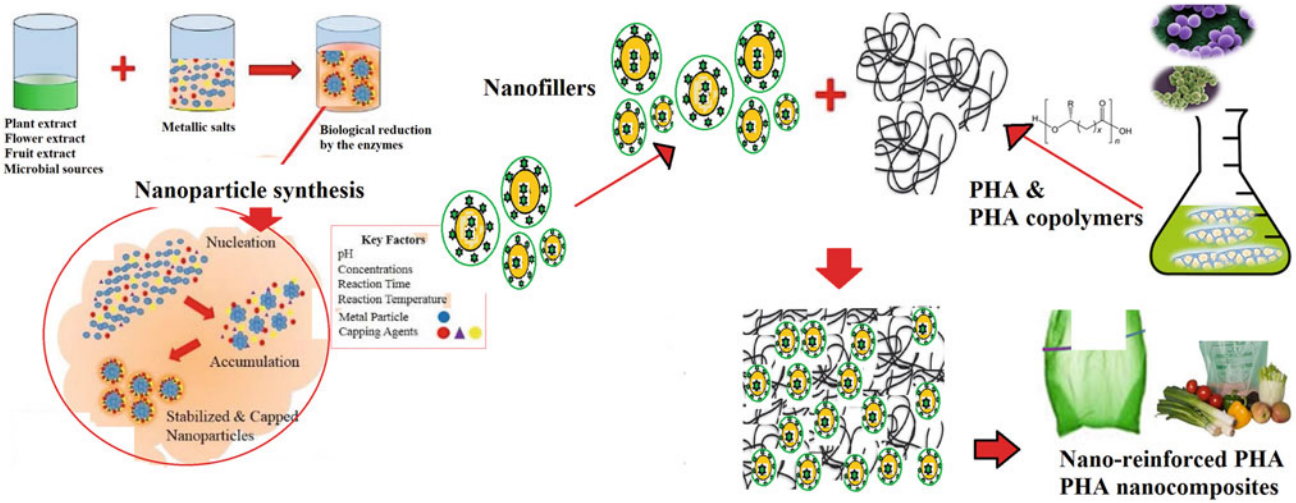

Fig. 1.7 Incorporation of nanomaterials into polyhydroxyalkanoates to fabricate multifunctional microbial plastics for food packaging applications

Table 1.3 Fabrication of multifunctional microbial plastics and their applications

Nano blended microbial plastics	Fabrication method	Properties	Applications	References
PHA nanocomposites				
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with Silica oxide nanoparticles (SiO_2) ($PHBHV/SiO_2$) nanocomposite	Solution casting technique	Improved thermal, mechanical, barrier, and antibacterial properties. Enhanced biodegradability and biocompatibility	Antibacterial food packaging	Ojha and Das (2020a, b)
Polyhydroxyalkanoates (PHA) with tailor-made long alkyl chain quaternary salt (LAQ) functionalized graphene oxide (GO-*g*-LAQ) (PHA/GO-*g*-LAQ)	Compounding PHA with GO-*g*-LAQ	Multifunctional and robust PHA nanocomposites with superior gas barrier, heat resistance, and inherent antibacterial properties	Functional material for food packaging	Xu et al. (2020)
Poly (3-hydroxybutyrate) (PHB) based calcium-iron layered double hydroxide (Ca-Fe LDH) nanocomposites loaded with norfloxacin (NFX) as a model drug (PHB/PEG/3NFX LDH)	Impregnation method and In vitro drug release assay	Sustained drug release and high bactericidal activity. Highly biocompatible and biodegradable	Potential use in antibacterial therapy, drug delivery	Kumari et al. (2020)
Poly (3-hydroxybutyrate-*co*-3–6% hydroxyvalerate) (PHBV) with Nanodiamond (nD) and nano hydroxyapatite (nHA) loaded with Vancomycin (VC). (PHBV/nHA/VC/nD-R) or (PHBV/nHA/VC/nD-SD)	Injection molding. Compounded using a rotary evaporator (PHBV/nHA/VC/nD-R) or a spray-dryer (PHBV/nHA/VC/nD-SD)	Combined antibacterial properties (bioactivity) with sustained drug release, higher glass transition temperature, and stronger interface	Promising for the treatment and prophylaxis of bone infection	de Almeida Neto et al. (2019)
		Enhanced flexural elastic modulus, good adhesion and growth of cells on the specimens, noncytotoxic and non-cytostatic behavior	Bone defect filling material	

(continued)

Table 1.3 (continued)

Nano blended microbial plastics	Fabrication method	Properties	Applications	References
PHBV/PBAT/nanoclay nanocomposite pellets	Melt extrusion process, compression molding, and cast film extrusion methods	Improved mechanical, barrier (oxygen and water vapor permeability) and rheological properties	Flexible packaging biomaterial	Pal et al. (2020)
PHB-chitosan (Cs)/multi-walled carbon nanotubes (MWCNTs) nanocomposite coating deposited on nano-bioglass (nBG)-titania ($nTiO_2$) (PHB-Cs/MWCNTs-nBG/$nTiO_2$) scaffolds	nBG/$nTiO_2$ scaffolds by foam replication method and coated with PHB-Cs/MWCNTs	Excellent hydrophilicity, compressive strength, and surface roughness	Bone tissue engineering	Parvizifard and Karbasi (2020)
		High proliferation and viability of MG-63 cells and higher secretion of alkaline phosphatase Biocompatible		
PHB-silver nanocomposite (PHB-AgNc)	Solution casting technique	Thermally stable, mechanically durable, and resistant to microbial attack	Food packaging applications	Jayakumar et al. (2020)
		Stable, polydispersive, and hydrophobic, excellent migration properties against food stimulants		
PHB with cobalt oxide (Co_3O_4) nanoparticles (PHB-Co_3O_4) bionanocomposite	In situ synthesis, Taguchi method	Improved structural and thermal properties	Pharmacological, medicinal, and dentistry packaging and medical applications	Safaei et al. (2020)
		Antibacterial activity against multidrug-resistant bacteria		
Cadmium sulfide (CdS) nanoparticles/PHB (CdS/PHB) nanocomposite	Chemical precipitation method and solution casting technique	Improved thermal properties	Packaging material	Riaz et al. (2020)
Poly-3-hydroxyoctanoate (PHO) with vinyl trimethoxy silane grafted sepiolite (VTMS-g-Sep) to formulate sepiolite/poly-3-hydroxyoctanoate (Sep/PHO) nanocomposite	Melt blending technique	Enhanced thermal and mechanical properties	Excellent packaging material	Masood et al. (2019)
		Excellent biodegradability		

Zirconium oxide-PHB (ZrO_2/PHB) or zirconium hydroxide-PHB ($Zr(OH)_4$) composite	Solution casting technique	Excellent thermal properties	Food packaging applications	da Silva et al. (2019)
Bacterial cellulose-based nanopapers (NPs) with medium-chain-length polyhydroxyalkanoate (MCL-PHA) (NP/PHA-e) nanocomposite	Valorization of apple waste Coating with impregnation method	Improved hydrophobicity, antioxidant, and free radical scavenging capacity	Potential active apple waste-derived multicomponent packaging film	Urbina et al. (2019)
		Enhanced elastomeric properties		
		High transparency and low oxygen permeability		
PHB/zinc sulfide (ZnS) (PHB/ZnS) nanocomposite	Chemical precipitation method and solution casting technique	Enhanced heat resistance and thermal properties	Packaging applications	Riaz et al. (2018)
PHB/copper sulfide (PHB/CuS) composite	Immobilization, aqueous to organic (hydrosol to organosol) phase transfer method	Photocatalytic degradation	Photocatalytic application	Ismail et al. (2018)
PHB with zinc oxide (ZnO) nanoparticles (PHB/ZnO) nanocomposite	Solution casting technique	High mechanical and barrier strength, antibacterial activity	Food packaging material, pharmaceutical drugs, capsules coating	Díez-Pascual and Diez-Vicente (2014)
PHA-silver nanoparticles (SNP) (PHA-SNP)	Dispersed colloids of PHA using sodium borohydrate ($NaBH_4$) as reducing agent	Enhanced the stability of colloidal silver nanoparticles	Biomedical and packaging applications	Phukon et al. (2011)
PHB-functionalized MWCNTs into a chitosan matrix (PHB-MWCNTs-chitosan) membrane	Filtration process and solution casting technique. Pervaporation process of 1,4-dioxane dehydration	High permeation flux and selectivity towards water Improved dispersion and biocompatibility	Biomembrane in biomedical applications	Ong et al. (2011)
PHA-polymer hybrids/blends				
P3HB with poly (ethylene glycol) (PEG) (PHB/PEG) hybrid	Simple blending method	Reduced roughness, increased hydrophilicity, protein adsorption, and higher biocompatibility	Biomedical applications	Israni et al. (2020)
		Enhanced thermal and mechanical properties		

(continued)

Table 1.3 (continued)

Nano blended microbial plastics	Fabrication method	Properties	Applications	References
Polylactide/polyhydroxyalkanoate/nano-fibrillated polytetrafluoroethylene (PLA/PHA/PTFE) composites	High-pressure foam molding (FIM) process combined with mold-opening technique	Feasible bio-foams with high expansion ratio and a desirable cellular structure High-molecular chain entanglements and enhanced impact strength	Biomedical applications	Lee et al. (2020)
Functionalized bacterial cellulose (BCA) with PHBV (BCA/PHBV) blended scaffold	Simple impregnation method. Highly hydrophilic BC was surface functionalized by amination (BCA) to improve its affinity to PHBV	Enhanced thermal and compression strength Good stability, easy diffusion of water and nutrient exchange/transport, nano-sized fibrous network with interconnected open porosity, excellent biocompatibility	Promising for tissue engineering scaffolds and regenerative medicine	Oprea et al. (2020)
Candida antarctica lipase B mediated functionalized poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-*co*-3HV)) with ascorbic acid (P (3HB-*co*-3HV)-ascorbic acid) blend	Esterification	Lower degree of crystallinity, higher thermal degradation temperature and hydrophilicity Excellent scavenging activity and biodegradability	Antioxidant active biomaterial, biomedical applications	Bhatia et al. (2018)
Poly(ε-caprolactone) (PCL) composites reinforced by PHBV fiber (PCL-PHBV) hybrid	Melt compounding	Enhanced tensile strength and storage modulus Improved thermal stability and biodegradability	Packaging material	Ju et al. (2014)
Epoxy networks of PHA with isosorbide derivative blends	Photoinitiated cationic ring-opening polymerization in the presence of diaryliodonium salt (I 250)	Higher degradability and thermal stability	Biomedical applications	Lorenzini et al. (2015)

PHA/silsesquioxane blend	Sol-gel process, conventional acidic, basic hydrolysis, UV curing in the presence of cationic photo-initiator	High crystallinity, thermal stability, photocatalytic properties	Biosensor, medical applications	Lorenzini et al. (2014)
PHA nanoparticles				
Poly-3-hydroxybutyrate-co-hydroxyhexanoate nanoparticles (PHBHHx-NPs) within whey protein (WP) (PHBHHx-NPs-WP) scaffold	Solvent-evaporation technique	Enhanced mechanical properties, excellent extensible materials preserving their mechanical resistance	Food packaging bioactive film	Corrado et al. (2020)
		Enhanced barrier properties towards O_2, the permeability to both water vapor and CO_2		
Epirubicin (EPI) loaded EPI-PHB-PEG and EPI-PHBV-PEG nanocarriers	Nanoprecipitation method	Ideal drug nanocarriers	Drug delivery	Perveen et al. (2020)
		Excellent antibacterial properties		
Carboxylated MCL-PHA nanosuspension	Carboxylation using mercaptoundecanoic acid	Enhanced stability of colloidal MCL-PHA	Biomedical nanomaterial	Peprah et al. (2018)
PHB-curcumin encapsulated nanoparticles	Nanoprecipitation method using dichloromethane as solvent.	Sustained drug release carrier	Drug delivery	Senthilkumar et al. (2017)
Amphiphilic P(3HB-*co*-3HV) or P(3HB-*co*-4HB) with monomethoxy poly(ethylene glycol) (mPEG) (PHA–mPEG) copolymeric nanocontainers	Emulsification–solvent evaporation technique, diblock copolymers formulated by chemical coupling of P(3HB-*co*-3HV) or P(3HB-*co*-4HB) to mPEG through transesterification reaction	Enhanced hydrophobic PHA segments buried in the core and could be used as safe carriers for the controlled release of a variety of hydrophobic drugs	Drug delivery	Shah et al. (2010)

Nanofibers with unique dimensions and structures, including glass, carbon, cellulose fibers, and carbon nanotubes are being applied as nanofilling agents for microbial bioplastics (PHAs). Inorganic nanofillers such as nano clay, silica, double-layered hydroxides, cellulose nanowhiskers (CNW), and multi-walled carbon nanotubes (MWCN) fuel the crystallization rate, thermal stability, and stiffness of the PHA nanocomposites. It was observed that the crystallization rate depends on rising or reducing the concentration of different nanomaterials. A high crystallization rate was observed at a low concentration of nanomaterials. In contrast, the crystallization rate was declined at a high concentration of nanomaterials (Bondeson et al. 2006; Ten et al. 2012). Another microcrystalline cellulose (MCC) or needle-like nanofibers is CNW that occurs in the cell wall of various plants and can be obtained using the sulfuric acid hydrolysis method (Bondeson et al. 2006). CNW strengthens PHBV with strong links for better thermal, mechanical and crystalline properties. It is an efficient PHBV nucleating agent, investigated by polarized optical microscopy (POM) analysis. Other aspects such as toughness, Young's modulus, and tensile strength of PHBV exhibited improved material properties with the green application of CNW/PHBV nanocomposite (Ten et al. 2010). In another study, cellulose nanocrystals (CNCs) were incorporated with PHBV through a solution casting method to enhance thermal stability. It was observed that both nucleation and confinement effects were depended on the CNCs concentration, leading to rise or reduction in the crystallization rate of CNCs/PHBV nanocomposites. Higher crystallization temperature and lower nucleation rate were observed in spherulite structured CNCs/PHBV green nanocomposites on increasing the concentration of CNCs. Thus, blends of these pure green composites (cellulose nanocrystals) with microbial plastics/polymers have been attracted the research interest as these nanoblends have similar features for biodegradation with high thermal and specific strength as compared to synthetic polymers (Ten et al. 2012) (Table 1.3).

1.9.2 PHA-Polymer Hybrids

Microbial bioplastics such as PHBV and PHB can be hybridized with other polymers including gelatin, chitosan, starch, soy protein isolate (SPI), cellulose acetate butyrate, cellulose, poly (butylene adipate-*co*-terephthalate) (PBAT), poly (lactic acid) (PLA), poly (propylene carbonate) (PPC), poly (caprolactone) (PCL), PEG poly (butylene succinate) (PBS) and poly (ethylene succinate) (PES) (Qiu et al. 2003; Vogel et al. 2008; Chun and Kim 2000; Nagarajan et al. 2013; Zembouai et al. 2013; Nar et al. 2014) to formulate PHA polymer hybrid nanocomposites with advanced properties. PBS is a thermoplastic polyester, strong and highly flexible with strong thermal stability, which was blended with PHBV using the solution casting method. In situ compatibility of PHBV-g-PBS polymers was enhanced on incorporating Dicumyl peroxide (DCP) into PHBV/PBS and PHB/PBS. PBS particle size was compacted in PHBV and PHB biopolymers which lead to the enhancement in the mechanical properties (Miao et al. 2008; Ma et al. 2012, 2014).

PHB was blended with poly(β-1,4)-2-amino-2-deoxy-D-glucopyranose, the deacetylated product of chitin using trifluoracetic acid as co-solvent under continuous stirring at room temperature to formulate PHB-chitosan blend. Besides, incorporation of titanium dioxide (TiO_2) nanofillers into PHBV/PCL using melt-mixed method resulted in improved thermal and mechanical properties of PHBV/PCL/TiO_2 hybrid (Mofokeng and Luyt 2015).

1.9.3 PHA Nanoparticles

Another approach which has been introduced was the synthesis of polymeric nanoparticles (PNPs) in the form of nanospheres or nanocapsules. PNPs are proved as a supreme ecofriendly nanocarrier for bioactive compounds aiming at the diseased cells with narrow side effects. The surface of PNPs can easily trap or encapsulate the drugs and carry them to the wound site. These engineered PNPs can be easily modified to treat the specific type of therapy as well as to carry specific drugs. Microbial bioplastics with similar biological and physicochemical properties are found to be noble candidates of PNPs against petrochemical synthetic polymers (Din et al. 2017). PNPs can be synthesized using different methods such as nano-precipitation and mini-emulsification. Nano-precipitation is a process where the target polymer is dissolved in an organic solvent and steadily supplementing to the non-solvent solution through a modifier. The nanoparticles (NPs) are formed at a phase of dispersing polymer-solvent with non-solvent in the aqueous medium. The increasing stress between the two phases, leading to the high surface area and precipitation of NPs. However, in the case of the mini emulsification method, the diffusion of polymer-solvent was done using a solution of water and surfactant followed by ultrasonication to produce emulsified NPs. The target drugs can be conjugated to NPs by evaporating the solvent using heat or by dispersing drugs with the polymer (Choiniere 2015).

1.10 Applications of Advanced Microbial Bioplastics

Multifunctional, high-performance, or advanced PHA nanocomposites with antibacterial properties have been developed by modifying PHA into PHA bionanocomposites, thereby extending their applications in fragile environments (Gumel and Annuar 2014). The improvement in the physical, thermal, mechanical, barrier, and flammability properties of PHA bionanocomposites has been investigated by different instrumental analysis such as Fourier transform infrared spectroscopy (FT-IR), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) spectroscopy, X-ray powder diffraction (XRD), atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), gel permeation chromatography (GPC), thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), pyrolysis combustion flow calorimetry (PCFC) and dynamic mechanical analysis (DMA). Today, the nano-blended or nano-impregnated PHAs exhibited enhanced thermal, mechanical, barrier, and antibacterial properties as compared to the neat PHAs. PHA bionanocomposites are expected to contribute significantly to the food packaging market soon providing multiple functionalities (Ojha and Das 2020b). There are various applications of advanced microbial plastics in the field of biomedical, bioengineering, and packaging industry which are discussed below and tabulated in Table 1.3.

1.10.1 PHA Nanocomposites for Scaffolds, Tissue Engineering, and Nanocoatings

There are reports where PHA nanocomposites including poly (3-hydroxyoctanoate-*co*-3-hydroxyundecenoate) (PHOHUd), poly (3-hydroxybutyrate-*co*-3-hydroxyoctanoate) (PHBHO), and poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) have been developed by reinforcing nanofillers such as silsesquioxane, bioglass, Cloisite 30B, kaolinite, clay, zein electrospun, and layered silicate for various applications in the field of formulation of scaffold for fibroblast cells, biosensor, cancer drug delivery, and bone tissue scaffold, respectively (Gumel and Annuar 2014).

Development of biomimetic materials is one of the most important challenges in tissue engineering research. An active scaffold was investigated by incorporating titanium dioxide (TiO_2) nanoparticles on the electrospun mats of PHBV by electrospinning technique. An increase in fiber diameter with the increased concentration of TiO_2 was observed in the nanocomposites fibers. The nanocomposites fibers showed excellent biocompatibility and improved cell proliferation which proved that TiO_2 nanoparticles were not cytotoxic for cells (Braga et al. 2018). The findings indicated that PHBV-TiO_2 fibrous mats (scaffolds) could be utilized as a good substrate for tissue regeneration.

In another study, photoluminescence (PL) analysis of polyhydroxybutyrate-*co*-valerate (PHBV)-zinc oxide (ZnO) nanocomposite fibers was investigated. The PL study revealed the quenching of visible emission in the nanocomposite fibers. Enhanced UV to visible emission (I_{uv}/I_{vis}) intensity was found in the PHBV-ZnO nanocomposite fibers as compared to pure ZnO nanoparticles. The findings revealed that PHBV-ZnO nanofibrous mats are self-supported which could provide potential applications in biomedical imaging and optoelectronic devices (Naphade and Jog 2012).

One of the valuable biomaterials for tissue engineering and regenerative medicine is bacterial cellulose (BC) sponges due to their nano-sized fibrous network with interconnected open porosity and biocompatibility. However, their poor mechanical properties and instability in the physiological environment are the main issues that need to be fulfilled in order to develop suitable three-dimensional (3D) scaffolds for tissue formation (Oprea et al. 2020). Recently, a polyester PHBV was impregnated into BC sponges using the impregnation method to improve its properties for biomedical application. To improve its affinity to PHBV, a highly hydrophilic BC was surface functionalized by amination (BCA). SEM microscopic images showed the formation of a tridimensional honeycomb ordered structure with uniform micrometer pores which confirmed the deposition of PHBV on the BC nanofibrous network. Better thermal properties and compression strength were observed for BCA/PHBV nanocomposite sponges as compared to the neat PHBV, due to the increased interactions between the PHBV and the aminated cellulose substrate. Swelling tests revealed that BC and BCA sponges were completely fragmented in 90 min of incubation in phosphate-buffered saline, but excellent stability was obtained in a simulated physiological environment after impregnation with PHBV. Higher swelling stability was observed for BC/PHBV (1200–2400%) and BCA/PHBV (700–1200%) sponges, which are quite enough to allow the transport of nutrients and diffusion of water. Thus, the improved properties of BC/PHBV and BCA/PHBV nanocomposite sponges lead to a promising option for tissue engineering scaffolds (Oprea et al. 2020).

Also, PHB-Chitosan (Cs)/MWCNTs nanocomposite coating accumulated on nano-bioglass (nBG)-Titania ($nTiO_2$) scaffolds were fabricated by foam replication method. The compressive strength of the PHB-Cs/MWCNTs scaffolds coated with 1.0 wt% MWCNTs was increased up to 30-fold compared to $nBG/nTiO_2$ scaffold. The PHB-Cs/MWCNTs scaffolds showed an increase in the percentage of interconnected porosity, surface roughness, and apatite-like formation on the coating with 1.0 wt% of MWCNTs with PHB-Cs. PHB-chitosan/MWCNTs nanocomposite coating leads to higher secretion of alkaline phosphatase, thermal properties, excellent proliferation and viability of MG-63 cells (Parvizifard and Karbasi 2020).

Besides, multifunctional bioglass poly (ester-urethane) nanocomposite scaffolds based on 1,6-hexamethylene diisocyanate-trimer, P(3HB), and silica nanoparticles were fabricated using sodium-acetate in situ particulate leaching technique. The nanocomposite could be used both as a candidate for tissue engineering and as agricultural mulch films for restoring contaminated soils. The scaffolds with salt-leached induced porosity only affected the adhesion but not the biocompatibility of the human mesenchymal stromal cells. The nanocomposite allowed strain *Azospirillum brasilense* to form biofilms in a stressful environment. Furthermore, the bacteria remained viable and metabolically active for up to a month without supplying any carbon or nitrogen source (González-Torres et al. 2020).

The currently exiting curing techniques have become ineffective towards healing wounds; this leads to the immense need for suitable biocompatible responsive biomaterials, which could heal the wounds quickly. Recently, a PHA-based antibacterial mat was reported which was fabricated by incorporating green graphene-decorated silver nanoparticles (GAg) into the fibers of poly (3-hydroxybutyrate-*co*-hydroxyhexanoate) (P3HB-*co*-HHx) via an electrospinning technique. The antibacterial property of PHA/GAg against gram-negative *Escherichia coli* (*E. coli*) and gram-positive *Staphylococcus aureus* (*S. aureus*) was examined, which showed a significant reduction of *E. coli* and *S. aureus* as compared to PHA-reduced graphene oxide (rGO) or bare PHA within 2 h of incubation period (Mukheem et al. 2018). The results showed that PHA/Gag could be applied as an efficient and faster wound healer bandage for biomedical applications.

1.10.2 PHA Nanocarriers for Cancer Therapy and Drug Delivery

One of the essential goals in cancer treatments is healing the cancer cells at the malign sites without affecting the healthy cells. Recently, the PHAs nanoparticles (PHANPs) which are biocompatible, nontoxic, and naturally degradable by nonspecific lipases and esterases have attracted the research interest for biomedical applications (Din et al. 2017). For the treatment of breast cancer cells, the linkers are connected to the functional moieties of PHANPs with other folate functional groups to reduce the overexpression of folate receptor α. For fluorescence imaging and tracing of live and dead cancer cells, functional groups of fluorescent tags are attached to PHANPs to trace and medicate the affected area. PHANPs attached to the MT3 line of breast cancer cells bearing fluorescent tags had been used as nano-tracer during the recovery of cancer cells. Poly (3-hydroxyoctanoate-*co*-10-undecanoate) nanoparticles (PHOUNPs) blended with PHO-N3 and Azide terminated PHA is another compound that has been applied in the therapy of cancer and drug delivery. The nontoxic PHOUNPs showed a significant impact on treating the A459 lung carcinoma cells through direct assay and for drug delivery on A549 cell line by indirect assay (Shah et al. 2011; Kilicay et al. 2016).

PHANPs are reported as an excellent nanocarrier for controlled drug release and enhanced therapeutic availability. For drug delivery systems, the size and zeta potential are two important aspects that must be measured while synthesizing in PHANPs. PHANPs can be used as a therapeutic nanocarrier by encapsulating the drug and carry it safely to the diseased prone area without damaging the healthy cells. Besides, PHANPs are highly capable of loading and controlled release of drugs as a safe nanocarrying agent. In a report, PHANPs were conjugated with levofloxacin using a triple emulsion method, and the in vitro levofloxacin release was investigated through the dialysis method. Levofloxacin-loaded PHANPs had showed improved solubility, sustainability, and therapeutic availability as compared to bare levofloxacin. Besides, 90% controlled drug release was observed until 22 h after the treatment (Umesh et al. 2018). In another report, an amorphous amphiphilic block copolymer, P(3HV-*co*-4HB)-monomethoxy poly(ethylene glycol) (P (3HV-*co*-4HB)-mPEG) was synthesized by blending poly(3-HV-*co*-4-HB) with mPEG using bis (2-ethyl hexanoate) tin catalyst coupled via transesterification reaction. The controlled release of chemotherapeutic cisplatin-loaded PHANPs was investigated using microscopic observations that revealed the internalization of cisplatin-loaded PHANPs into the tumor cells. Besides, the cisplatin-loaded PHANPs were found to suppress the tumor cell growth and compel the tumor cells into the process of apoptosis (Shah et al. 2012).

1.10.3 PHA Nanocomposites as Smart and Active Packaging Material

Recently, biogenic nanoparticles have been investigated to use as smart green, biocompatible, and active reinforcing nanofiller agents. A recent report by Zare et al. (2019) investigated a smart fortified PHBV-CS biopolymer reinforced with zinc oxide-silver nanocomposites (ZnO-Ag NCs) for the advanced shelf life of food packaging material. ZnO-Ag NCs were prepared using *Thymus vulgaris* leaf extract as a stabilizer and reducing agent. The ZnO-Ag NCs were reinforced into poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate)-chitosan (PHBV-CS) to fabricate the PHBV-CS/ZnO-Ag nanocomposite via solvent casting method. The ZnO–Ag NCs act as a nanoactive agent which helps in food preservation by controlling its spoilage from contaminants, and as a result, its shelf life was enhanced. The PHBV-CS/ZnO-Ag nanocomposite was investigated as a potential sensory evaluator of refrigerated chicken breast, enhanced thermal, mechanical, and barrier properties. Antimicrobial activity was shown which suggested a prospective replacement of traditional synthetic plastics currently being used for poultry and food items packaging (Zare et al. 2019). A recent report by Ojha and Das (2020b) investigated PHBV/SiO_2 nanocomposites which were formulated by reinforcing biogenic silica nanoparticles (bSNPs) into *Wickerhamomyces anomalus* synthesized PHBV via the solution casting method. The PHBV/SiO_2 nanocomposites revealed considerable enhancement in the barrier, heat resistance, thermal and mechanical properties as compared to the pure PHBV matrix. The improvement in the material properties of PHBV/SiO_2 nanocomposites was due to the uniform dispersion of bSNPs and its strong hydrogen-bonding interactions with the PHBV matrix. Besides, excellent biocompatibility against L929 mouse fibroblast cells and antibacterial activity against *S. aureus* and *E. coli* of PHBV/SiO_2 nanocomposites was observed upon increasing bSNPs concentration (Ojha and Das 2020b). The biodegradable PHBV/SiO_2 nanocomposites revealed excellent antimicrobial and biocompatible properties and proved as an efficient active packaging material which could extend the shelf-life of food and minimize the spoilage caused by microorganisms. Hence, the biodegradable PHBV/SiO_2 nanocomposite could serve as an eco-friendly and antibacterial food packaging material.

Many antibacterial nanoparticles have been reinforced to PHAs to formulate antibacterial packaging materials. There are reports where ZnO-reinforced PHBV bionanocomposites with antimicrobial function were investigated for food packaging (Díez-Pascual and Diez-Vicente 2014). PHBV/ZnO films showed antibacterial activity against human pathogen *Escherichia coli* and *Staphylococcus aureus*. The nanocomposites also showed excellent stiffness, impact strength, toughness and lower water uptake, and oxygen and water vapor permeability due to the strong interfacial adhesion formed between the matrix and nanofiller via hydrogen bond formation. In addition, enhanced tensile strength, thermal, and barrier properties were also obtained. A similar report investigated by Yu et al. (2010) demonstrated the formulation of PHBV/ZnO nanofibers by electrospinning method. These sustainable nanomaterials with antimicrobial function showed great potential as an alternative to synthetic plastic packaging materials, especially for use in food and beverage containers and disposable applications like cutlery or overwrap films (Yu et al. 2010).

In another study, cellulose nanocrystals (CNC) and ZnO were incorporated into the PHBV to fabricate PHBV CNC-ZnO nanohybrids via simple solution casting for possible use as antibacterial biomedical materials (Abdalkarim et al. 2017). The nanocomposites exhibited an excellent antibacterial (95.2–100%) activity for *S. aureus* and *E. coli* and 9–15% degradation after 1 week. The incorporation of CNC-ZnO resulted in a positive effect on hydrophilicity and barrier properties. Most importantly, the nanocomposites showed significant improvement in Young's modulus (183.1%), tensile strength (140.2%), and the thermal degradation temperature on incorporating 10 wt% of CNC-ZnO. A possible mechanism for utilizing CNC-ZnO nanofillers on PHBV hydrolytic degradation was noted which was found to be beneficial to develop the high-performance nanocomposites with modulated degradation rate for antibacterial biomaterials. Modified PHBV by impregnating chitin nanocrystals through chlorination was investigated to enhance the thermal properties of PHBV (Wang et al. 2012).

PHB-silver nanocomposite (PHB-AgNc) was reported to be stable, polydispersive, and hydrophobic. It exhibited a degradation temperature of 340 °C with a significant antimicrobial resistance against common food pathogens such as *Pseudomonas* spp. and *E. coli*. Mechanical properties such as Young's modulus, tensile strength, and elongation at break were found to be 1.305%, 35.42, and 1.058 N/mm^2, respectively. The migration properties of PHB-AgNc films were tested for various food stimulants, and the values obtained were less than the overall migration limit established for food contact materials which proved that the PHB-AgNc film could serve as efficient food packaging material (Jayakumar et al. 2020). In another study, the impact of different dosages of gamma irradiation on the biodegradable and physical properties of PHB/sepiolite (PHB/SP) nanocomposites was investigated. A proper network formation was observed in the SEM micrographs of nanocomposite films. Formation of agglomerates, pits, and biofilms on the surfaces of irradiated nanocomposite films was observed after in vitro and soil burial. SEM micrographs revealed better biodegradability as compared to unirradiated nanocomposite films (Masood et al. 2018). These findings proposed the potential application of the PHB/SP nanocomposite as a biodegradable food packaging material.

Omri et al. (2020) reported a novel nanocomposite which has been formulated from ethylene-*co*-vinyl acetate copolymer (EVA) and PHBV in combination with small amounts of Fullerene (C60) A significant increase in the thermal degradation pattern of nanocomposite at low loading (0.3, 0.5, and 0.7 wt%) dispersion of C60. The oxidation induction time values of nanocomposites were remarkably increased with the increase of C60 amounts. Furthermore, the oxidation induction time of EVA/PHBV/C60 (0.3 wt%) was 1643 s higher than that of the unfilled EVA/PHBV blend. The flammability properties showed that the addition of C60 could prolong the time to peak heat release rate (pHRR) of around 30 °C compared to EVA/PHBV blend. It was demonstrated that C60 is an inhibitor of the thermal and thermo-oxidative degradation of EVA/PHBV blend.

In addition, there are reports where PHA has been compounded with tailor-made long alkyl chain quaternary salt (LAQ) functionalized graphene oxide (GO-g-LAQ) that could disperse in the PHA matrix uniformly and improve the interfacial adhesion between GO and PHA due to the hydrophobic nature of LAQ. The modified PHA/GO-g-LAQ nanocomposites reported 99.9% antibacterial activity against Gram-positive and Gram-negative bacteria without any leaching. Besides, the oxygen barrier was found to be improved due to the condensed crystal structure of PHA and the impermeable property of GO sheets. Besides, the tensile strength, storage modulus, and heat-resistant performance of the PHA nanocomposites were significantly improved due to the enhanced crystallization capability. Therefore, the well-designed PHA/GO-g-LAQ nanocomposites could be ideal candidates for functional materials for food packaging applications (Xu et al. 2020).

In another study, PHBV/PBAT was blended with different concentrations of organically modified nanoclay (0.6, 1.2, and 1.8 wt%) using compression molding and cast film extrusion techniques. Firstly, the pellets of PHBV/PBAT/nanoclay nanocomposite were prepared using the melt extrusion method; then, nanocomposite pellets were used to fabricate films using compression molding and cast film extrusion method. The cast extruded PHBV/PBAT nanocomposite films showed improved oxygen and water vapor, i.e., barrier properties as compared to compression molded films because of the chain orientation during the high-speed stretching. The barrier and rheological properties of cast PHBV/PBAT/nanoclay (1.2%) nanocomposite film showed significant improvement in oxygen and water vapor permeability due to the excellent dispersion of nanoclay and its strong interfacial adhesion with the PHBV/PBAT matrix. Also, % elongation at break (567.6 ± 0.1) was highly improved as compared to that the PHBV/PBAT blend due to better stress transferability. Hence, the cast extruded nanocomposite film with 1.2% nanoclay was proved as a potential candidate to be used in flexible packaging (Pal et al. 2020). Besides, PHA nanocomposites such as PHB, PHB4HB, and PHBV have been developed by reinforcing nanofillers such as MWCN, chitosan matrix, and montmorillonite for packaging applications of pharmaceutical drugs, capsules, and food. Ajmal et al. (2018) reported the clay polymer nanocomposite which was fabricated using PHBV and vinyl triethoxy silane grafted sepiolite (VTES-g-SEP) by the solution casting method. The absence of voids and high interfacial interaction between PHBV and VTES-g-SEP was observed using SEM micrographs. Better biodegradability and thermal and mechanical properties of the clay polymer nanocomposites were observed as compared to the PHBV, which proved that it could be used in packaging applications.

1.11 Conclusion and Future Perspectives

Microbial bioplastic has gained considerable research interest and become a promising agent for an extensive range of applications due to its biodegradable, biocompatible, and thermoplastic nature. The recent development of advanced PHA nanocomposites and PHANPs can function as a reliable alternative to petrochemical synthetic plastics compared to other bioplastics. Nanocomposites of PHB, PHBV, and other PHA copolymers were proved as promising materials for use in the medical field such as tissue engineering, bone scaffoldings, surgical implants, and drug delivery for the healing of wounds and blood vessels. Blending of PHA and its copolymers with other biopolymers such as chitosan and cellulose impregnated with antimicrobial nanoparticles was proved as an excellent smart and active packaging biomaterial which can be utilized as a promising alternative to nonbiodegradable packaging material. There are more ways to explore some excellent eukaryotic extremophiles for optimization of the cost-effective PHA production parameters. Extensive studies can be carried out on the biogenic and herbal PHA synthesis for developing a multifunctional high-performance green microbial plastic in the future.

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General Structure and Classification of Bioplastics and Biodegradable Plastics

2

Erum Dilshad, Hasnain Waheed, Umar Ali, Arshia Amin, and
Iftikhar Ahmed

Abstract

The term "bioplastics" refers to chemically unrelated products that are manufactured exclusively or partially from renewable biomass sources such as agricultural products or microbes such as bacteria, yeast, and sometimes several nanometre-sized carbohydrate chains (polysaccharides). Bioplastics prepared from renewable means can be naturally recycled by biological processes, thus limiting the use of fossil fuels and shielding the environment. Biodegradable plastics are categorized as agro-polymers (starch, chitin, protein) and bio-polyesters (polyhydroxy-alkanoates, polylactic acid). Usually, foodstuffs with limited shelf life including fresh vegetables and fruits as well as those with long shelf life like cooked food items which are not in the need of increased oxygen supply are parceled using these bioplastics. Mainly, the enzymatic actions of microorganisms cause bioplastics polymers to decay into CO_2, H_2O, and other inorganic compounds. This current effort provides a general idea about bioplastics, aiming their production methods from biomass-based resources. Similarly, it will discuss the origin and classification of bioplastics. Furthermore, the details about the structure and components of bioplastics including polymers and the ways how these polymers are biochemically converted into bioplastics will also be reviewed. Moreover, different biopolymers currently under research will also be explored. With further improvements in the biopolymer area of

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research in the coming future, it can be more effectively utilized in various fields of life.

Keywords

Biopolymers · Bioplastics · Biodegradable · Classification · Biochemical synthesis · Renewable resources

2.1 Introduction

Bioplastic simply means the use of living organisms such as plants, animals, and microorganisms for the production of plastic. This type of plastic is different in many ways from fossil fuel-derived plastics. The bioplastic derived from biomass sources is also known as bio-based plastic. These are usually biodegradable because they are derived from renewable sources and can degrade by direct hydrolysis. A wide range of oil-based polymers are now utilized in many industries as packaging material. Plastic leftover is one of the most important issues in the present world because the plastic derived from fossil fuels is nondegradable and difficult to recycle, so this type of plastic is a major contributor to solid waste. To overcome the nondegradable plastic waste, scientists have now introduced bioplastics that are derived from living organisms (Kumar and Thakur 2017). According to the European Bioplastics Organization (EBO), bioplastics are referred to as plastics which are biobased and are biodegradable and/or compostable. These bioplastics are decomposed into carbon dioxide and water and biomass of inorganic nature under natural or unnatural conditions mainly by the degradation caused by the enzymes of microorganisms (Song et al. 2009). Furthermore, it is also required that these materials should be almost 50% organic in nature by composition. The developed polymer should be cut down during 6 months under-stimulated composting state by at least 90% of its weight/volume (Ezeoha and Ezenwanne 2013).

In recent years, bio-based polymers are extensively utilized for packaging purposes in various industries. The use of bio-based polymers has encouraged researchers to support ecological development because of diverse disposable options (Kumar and Thakur 2017). The major interests behind the production of bioplastics are mainly two in number: the first interest is the making bioplastic in bulk quantity, and the second is the proper disposal of bioplastic. Fossil fuel-based plastics are very slowly degraded in the environment that is why they are considered nonbiodegradable. There is a variety of biodegradable polymers such as lignin, starch, and cellulose that are actively involved in the production of bioplastics (Ross et al. 2017). The major drawback of the use of nondegradable plastics obtained from petrochemicals involves the emission and long-period accumulation of carbon dioxide in the environment. This accumulated carbon dioxide affects the ozone layer and is also a major contributor to climate change. Therefore, the world is seeking the sustainable use of plastic (bioplastic) that can be easily degradable and are environment-friendly (Tokiwa et al. 2009). To move toward a sustainable environment and prevent the disposal of plastic waste in the environment, the production of bioplastic has gained a lot of interest due to its high biodegradable ability. Bioplastics obtained from biomass or renewable resources include polyhydroxyalkanoate (PHA) and polylactic acid (PLA) in contrast to the plastics obtained from fossil fuels (like polybutylene succinate, PBS) that put a negative impact on the environment (Mekonnen et al. 2013). Although bioplastic is considered to be environment-friendly, it may have some limitations such as poor mechanical properties and high production cost. High production costs can be tackled by utilizing agriculture waste that is a low-cost renewable resource. Polylactic acid (PLA) is a type of bioplastic that has some unique properties like high tensile strength and modulus, but polyhydroxyalkanoate (PHA) lacks some mechanical properties when compared with polylactic acid (Tabasi and Ajji 2015).

These bioplastics can be degraded very easily in the environment and can be recycled easily. The degradation of polymer is completed in three steps in the environment: (1) biodeterioration, the microorganisms grow in or on the surface of the polymer, which changes the chemical, physical, and mechanical properties of the polymer; (2) bio-fragmentation, the microorganisms act on the large polymer unit and convert it into oligomers and monomers, the smaller units; and (3) assimilation, after the breakdown of polymer into smaller unit monomers, there is a release of a large amount of carbon, energy, and nutrient sources that are used by the microorganism and convert them into the water, carbon dioxide, and biomass (Lucas et al. 2008). Some factors actively involved in the degradation of plastics in the environment are the polymer chain, the chemical structure of the polymer, and the complexity of a polymer formula along with crystallinity. The specific functional group is selected and can be processed by using an appropriate enzyme. The polymers that have a more amorphous part, a shorter chain, and a less complex formula are more easily degraded by microorganisms (Massardier-Nageotte et al. 2006).

2.2 Types of Bioplastics

There are mainly three types of bio-based polymers which have been designed depending upon their source and manufacturing process (Ezeoha and Ezenwanne 2013): (a) polymers obtained from biomass, i.e., polysaccharides (starch and cellulose) and proteins (casein and gluten); (b) polymers obtained via renewable bio-based monomers. Polylactic acid is a good example here, which is obtained from monomers of lactic acid. The monomers themselves may be made via fermentation of carbohydrate feedstock and (c) polymers obtained from microorganisms. To date, bio-based polymers of this group consist primarily of polyhydroxyalkanoate, but advancement with bacterial cellulose is in progress.

2.3 Sources of Bioplastic

Plastic plays a very significant role in our daily life because there are a lot of things that we use today are directly or indirectly made up of plastic. But too much plastic use in our world makes plastic a major contributor to solid waste due to its nondegradable ability. That is why the world is seeking the alternative use of plastic that must be easily degraded and should be environment-friendly. So, bioplastics have their importance due to their degradable property and can be easily recycled. They are acquired from several biological foundations such as plants, microorganisms (bacteria), and algae. The plant and microbial products are the major contributors to the production of bioplastics (Reddy et al. 2003).

2.3.1 Plants as a Source of Bioplastics

The main plant source that is responsible for the production of bioplastic is starch. Starch is a naturally occurring polysaccharide consisting of two types of polymers: amylose and amylopectin. Starch is abundantly present in wheat, corn, sweet potato, barley, rice, etc. Along with starch, cellulose is also of key importance in bioplastic production. Thermoplastic starch is very common and widely used in the synthesis of bioplastic. The pure form of starch has a unique humidity-absorbing property; that is why pure starch is commonly used as bioplastic and is used in synthesizing drug capsules in pharmaceutical companies. Polylactic acid (PLA) is also a widely used bioplastic that is commonly obtained from cane sugar. There is a lot of genetic engineering practices that are being done on plants to get next-generation bioplastics (Rajendran et al. 2012).

2.3.2 Bacteria as a Source of Bioplastic

Bacteria are the most studied organism for the production of bioplastic. There are a lot of bioplastics that are directly or indirectly synthesized from bacteria. Many bacterial species contain intracellular polyhydroxyalkanoate (PHA) granules as a source of carbon and energy in their cells. The PHA that is extracted from gram-negative and gram-positive bacteria shows similar properties to polypropylene (Braunegg et al. 1998). *Bacillus megaterium* is an important bacterial species for the production of PHA because this bacterium contains 20% w/v of PHA content. The bacteria produce acetyl coenzyme A, which is converted into poly-3-hydroxybutyric acid (PHB) by the activity of three enzymes: acetoacetyl-CoA reductase, 3-ketothiolase, and PHB synthase (Ross et al. 2017).

2.3.3 Algal Sources

Algae are one of the diverse groups of organisms that range from unicellular to multicellular photosynthetic organisms. The by-products of many algae are used in the production of bioplastics. *Spirulina dregs* are microalgae commonly used in bioplastic manufacturing, but this alga is difficult to harvest. But macroalgae like seaweeds have many advantages over microalgae. The seaweeds produce high biomass, are cost-effective with the capability to grow up in a wide range of environments, and can be easily cultivated and harvested in a natural environment. Seaweeds produce polysaccharide that is commonly used in the field of microbiology, food technology, biotechnology, medicine, and now plastic industries. Bioplastic is now produced from the polysaccharide of seaweeds, this bioplastic is low-cost, environment-friendly, and nontoxic. The bioplastic derived from seaweeds has high tensile strength and much better in all aspects than the conventional plastic that is being used today (Rajendran et al. 2012).

2.4 Classification of Bioplastics

The bioplastics also known as biodegradable polymers are obtained from biomass, microorganisms, are petrochemicals and are biotechnologically derived. The bioplastics are majorly divided into two main categories that are agro-polymers and bio-polyesters (Fig. 2.1). Agro-based polymers are derived from natural renewable sources that may be polysaccharides, starch, cellulose, lignin, pectin, animal, and plant proteins and oils, while the bio-polyesters are obtained from microorganisms, petrochemicals, and biotechnological means. In agro-polymers, the main source is biomass that is obtained from plants and animals. Biopolymers that are derived from starch have many key features like renewability, high biodegradability, and good oxygen barrier property that make it more suitable than ordinary plastic (Thuwall et al. 2006). Most of the proteins that are obtained from animal and vegetable sources are used as a raw material for the production of bioplastics. Proteins are a well-known source used in the plastic manufacturing industries (Kumar and Thakur 2017).

2.4.1 Bioplastic from Biomass Products

Biomass is the material obtained from plants and animals, and these materials are used for the synthesis of bioplastic. The bioplastic obtained from biomass is also known as agro-bioplastics. The biomass products include polysaccharide, starch, cellulose, chitin, lignin, pectin, and proteins derived from plants and animals. These substances are directly obtained from plants and animals, and these are the major contributors to the production of various types of bioplastics (Kumar and Thakur 2017).

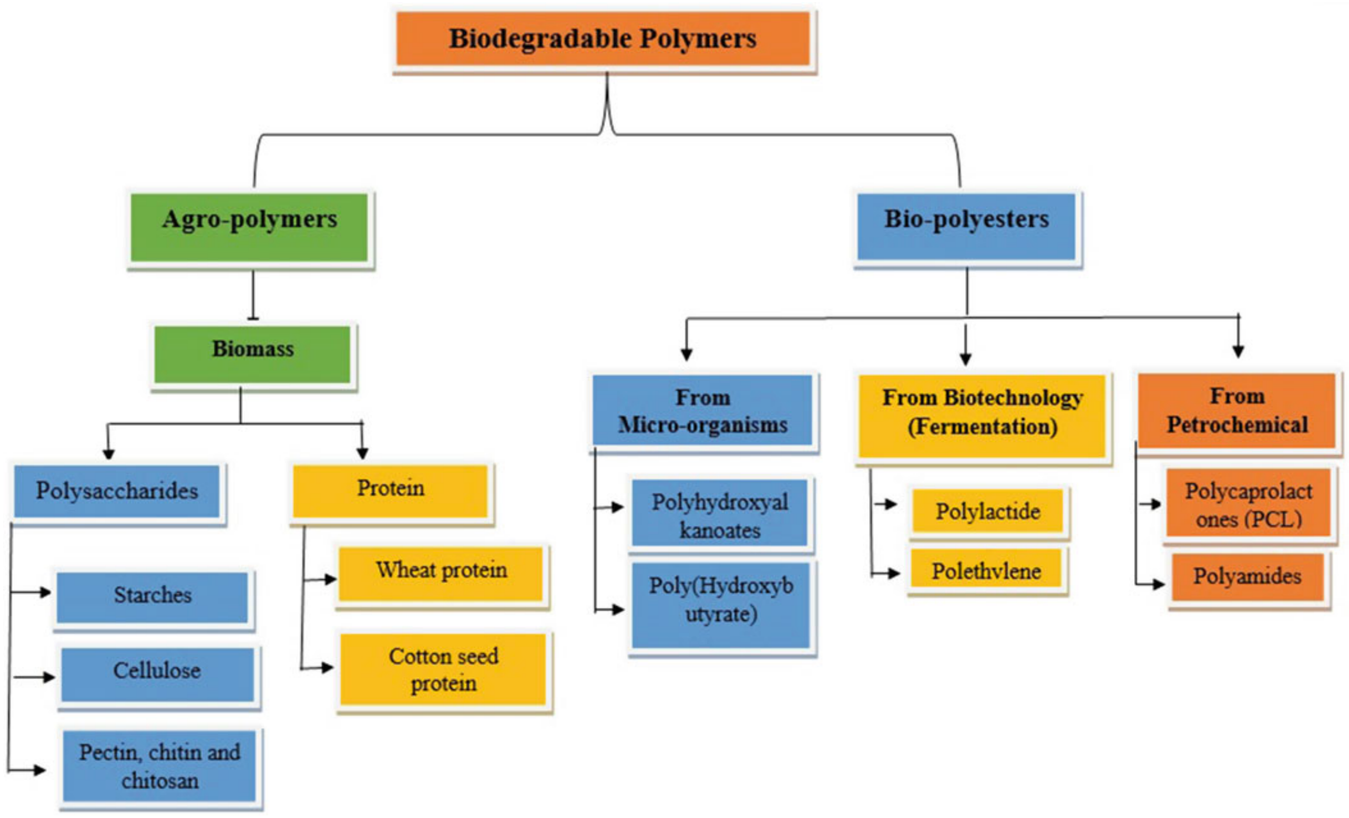


Fig. 2.1 Classification of biodegradable polymers (Kumar and Thakur 2017; Palanisamy et al. 2019)

2.4.1.1 Bioplastic-Based on Polysaccharide

There have been variously reported polysaccharides that are involved in the production of bioplastics. Polysaccharides are a long chain of carbohydrate molecules that bind with one another through glycosidic linkages. There are a lot of polysaccharides that have unique properties, which is why they are used in bioplastic production (Mali et al. 2002).

2.4.1.2 Bioplastic Obtained from Starch

Starch is one of the best-known polysaccharides that is formed by the combination of amylose and amylopectin (Fig. 2.2). The specific ratio of these two glucose-based polymers makes its unique physiochemical properties. If starch has a high content of amylose, this contributes to the film strength of the polymer, and if the starch contains branched amylopectin, then this will lead to a film with low mechanical properties. This low mechanical property of polymer can be upgraded by means of plasticizers such as glycerol and sorbitol (Mali et al. 2002). Different biopolymers that are manufactured by blending conventional polymers with granular starch will increase the strength, permeability of film, and water absorption and decreases the cost of production of the polymeric film (Kumar and Thakur 2017).

Bioplastic from the Modified Form of Starch

The starch molecules are manipulated for their physiochemical characteristics by adding the ester group. This manipulation improves the thermal stability, moisture absorption, and water vapor transmission rate and also increases the barrier properties for different gases. Starch has a good biodegradable property, which is why starch molecules are used in conventional polymers to increase its biodegradable ability. Scientists have successfully proved how starch mixed with a conventional polymer like low-density polyethylene to improve the biodegradable property (Albertsson and Karlsson 1995).

2.4.1.3 Bioplastic Obtained from Cellulose

Cellulose is naturally occurring biodegradable polysaccharides in plant cells. Cellophane is a chemically synthesized biodegradable material that is made by dissolving the cellulose in the mixture of carbon disulfide and sodium hydroxide and obtained product is cellulose xanthate (Fig. 2.3) which is further dipped into the acid solution (H_2SO_4), and the final product produced is cellophane film (Jabeen et al. 2015). Another method is adopted to obtain the derivatives of cellulose by the process of the esterification and etherification of the hydroxyl groups (Cyras et al. 2007). To make the cellulose as bioplastic material in the form of thermoplastic materials, further additives are added as cellulose diacetate and cellulose triacetate. These chemicals enhance the extrusion molding and laminating process, and finally, cellophane exhibits good film properties (Zepnik 2010).

2.4.1.4 Bioplastic Obtained from Pectin

Pectin is a linear biomacromolecule which is based upon the linear configuration of α-(1-4)-linked D-galacturonic acid. Many chemical changes are produced in the

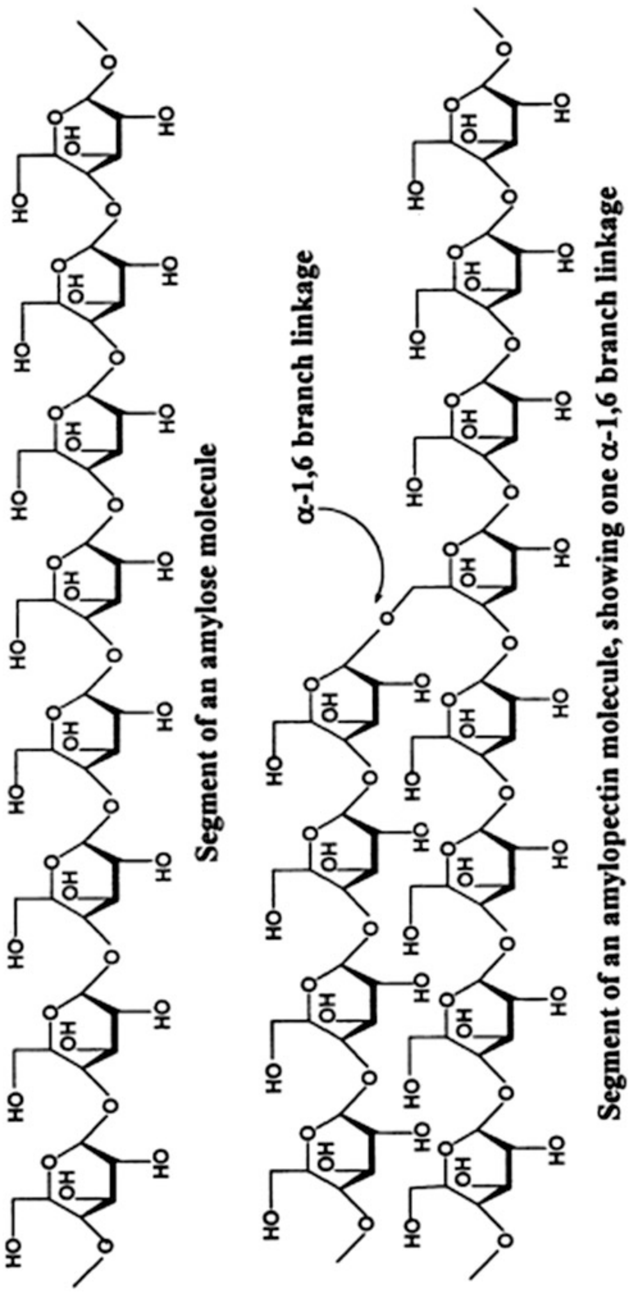


Fig. 2.2 Structure of starch, showing a segment of amylose and amylopectin (Robyt 2008)

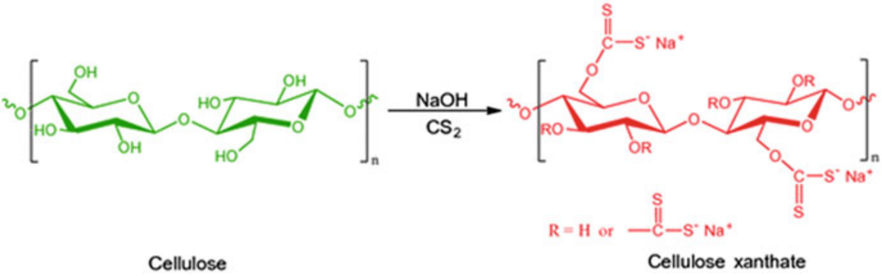


Fig. 2.3 Synthesis of cellulose xanthate from cellulose (Boy et al. 2018)

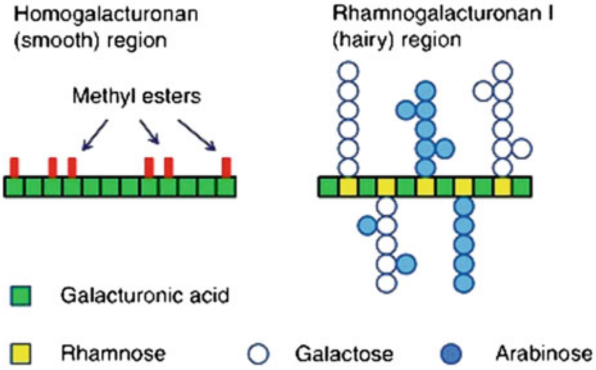


Fig. 2.4 Rhamnogalacturonan I and rhamnogalacturonan II as bio-polymers (Jaskolski 2013)

pectin by the partial replacement of monomer units like an α-(1-2)-L-rhamnose leading to a new structure rhamnogalacturonan I (Fig. 2.4). Rhamnogalacturonan II is the third type of new structure which is common, except highly for branched and complex polysaccharide structure (Fig. 2.4) (Thakur et al. 1997). In nature, ~80% esterification of the galacturonic acid carboxyl group is based on the methanol; this condition is based on the proportion of extraction. In food applications, the behavior of pectin depends upon the ratio of esterified and nonesterified galacturonic acid. Based on esterification, pectin is classified as high- and low-ester pectin (Malathi et al. 2014; May 1990).

2.4.1.5 Bioplastic Obtained from Chitin and Chitosan

Chitin is one of the most familiar agro-polymer after the cellulose in nature that is produced in rich quantity. It mainly exists in nature in the form of an orderly crystalline microfibril structure. Chitin is also the main component of the exoskeleton in many arthropods and also a component of the fungal and yeast cell wall (Rinaudo 2006). It is naturally occurring polysaccharides that are biodegradable, nontoxic, and biocompatible (Itoh et al. 2002). Flieger et al. (2003) identified that usually, the extraction of naturally occurring chitin is based on the crab crumb during

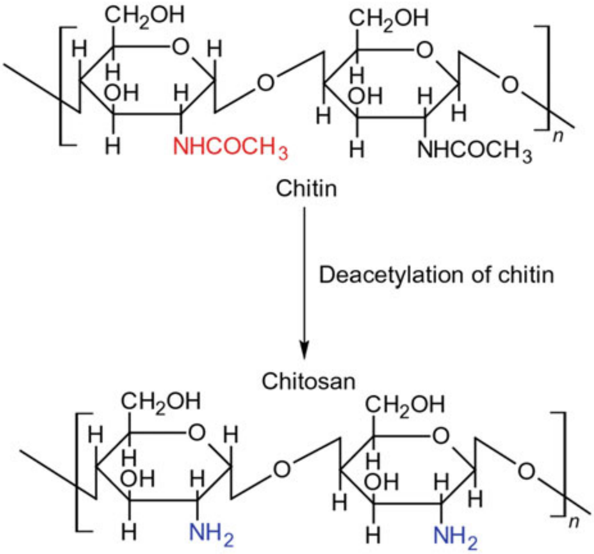


Fig. 2.5 Synthesis of chitosan from chitin (Królczyk et al. 2020)

pretreatment; dilute solution of sodium hydroxide (pH 13.5) is used for the prevention of microbial infection of the flesh of crab and protection of shell degradation. The isolated crushed shell passes into the rector for the further process where they are treated with hydrochloric acid (HCL) to gasify the materials (Flieger et al. 2003). In the third step of the process, produced chitin and liquefied proteins go into the washing procedure before ingoing into another NaOH solution, following a slightly high temperature. After completion of the whole procedure, 12% of chitin yield is obtained from crushed crab crumbs. Chitin is used to produce chitosan from various chemical processes (Fig. 2.5). In the process, chitin is washed and put under the boiling condition until acetate is removed from the molecules. After the hydrolysis, the resulted material chitosan is shifted for the following procedure, washed, dried, ground, weighed, and packed for sale.

2.4.2 Bioplastic Obtained from Proteins

Proteins have vast and empirical importance for the production of bioplastics. The main sources of protein are plants and animals that act as a raw material in the synthesis of bioplastics. Proteins play a significant role in thermosetting modifications of plastics due to their denaturation ability. The protein-based bioplastics are well-known for their high degradability and also make them a fast degrading polymer. A certain number of proteins have gained maximum attention due to their biodegradable polymer nature, but only a few of them have an impact due to their definite industrial scale-up, high assembly cost, and low product performance. There are certain plant proteins which are the main potential source such as com protein (zein), soy protein, and wheat protein (gluten) (Sorrentino et al.

2012). Some animal-based proteins also act as biodegradable polymers such as gelatin or collagen protein and casein. These are purely animal-based proteins. Some proteins inside the bacteria also play an effective enzymatic role such as lactate dehydrogenase, fumarase, and chymotrypsin (Itoh et al. 2002).

2.4.2.1 Bioplastic from Wheat Gluten Protein

There is a protein known as wheat gluten, a by-product that is produced from the bio-ethanol industry also used for packaging purposes in the baking industry. This wheat gluten has many advantages over other proteins like abundantly available, relatively inexpensive, and mechanical and biodegradation ability. Wheat gluten also has other unique properties such as gas barrier, film formation, and biodegradation ability. The properties of polymers developed from wheat gluten depend upon the two types of proteins: gliadins, and glutenins (Fig. 2.6). The wheat gluten is usually thermoplastic and it has been utilized for manufacturing natural fiber-based bio-composite and biofilms (Muneer et al. 2014).

2.4.2.2 Bioplastic from Cottonseed Protein

Cottonseed is also an important source of plant protein. It has a unique amino acid combination that increases its nutritional value and commonly used in cattle feeds but not as much used in nonfood industries. The protein-based bioplastics have low mechanical properties as compared to synthetic polymers. Its inferior mechanical properties are due to its hydrophilic nature, complex composition, and environmental sensitivity. To overcome these issues, protein requires different modifications like

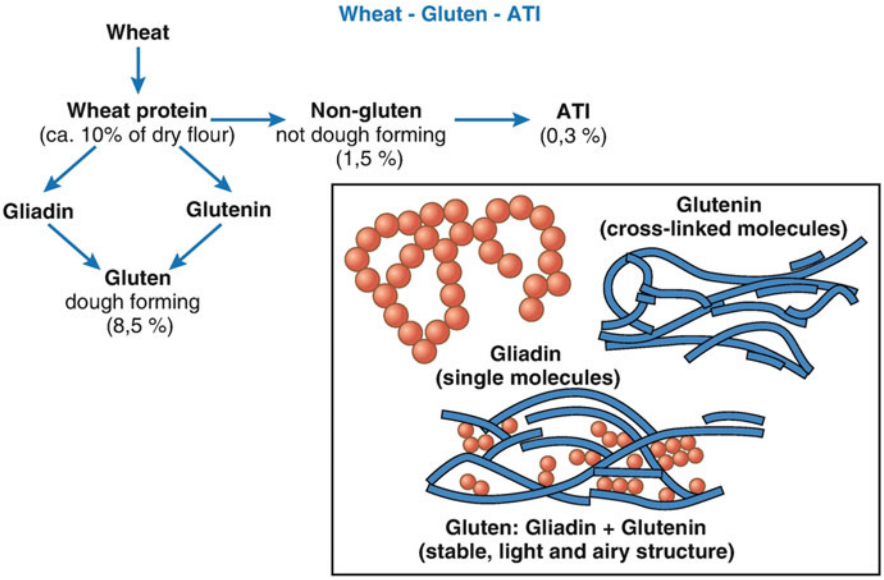


Fig. 2.6 An overview of wheat gluten composition (Schuppan and Gisbert-Schuppan 2019)

plasticization, denaturation, and cross-linking. Cottonseed protein is used to produce bioplastic. The protein obtained from cottonseed undergoes some modifications like denaturation and cross-linking with aldehyde and urea. This modified form of cottonseed protein is capable to produce bioplastics (Yue et al. 2012).

2.5 Bioplastics from Microorganisms

Bioplastic is a special type of biomaterial. They are usually polyester in nature and can be produced in microbes under different nutritional and environmental conditions. Most of the polyesters are produced inside the microbes under the lack of some important nutrients. They are usually a storage substance that is produced by microbes under stress conditions. The number of granules, size of granules, and physicochemical properties vary from species to species (Calero-Bernal et al. 2020).

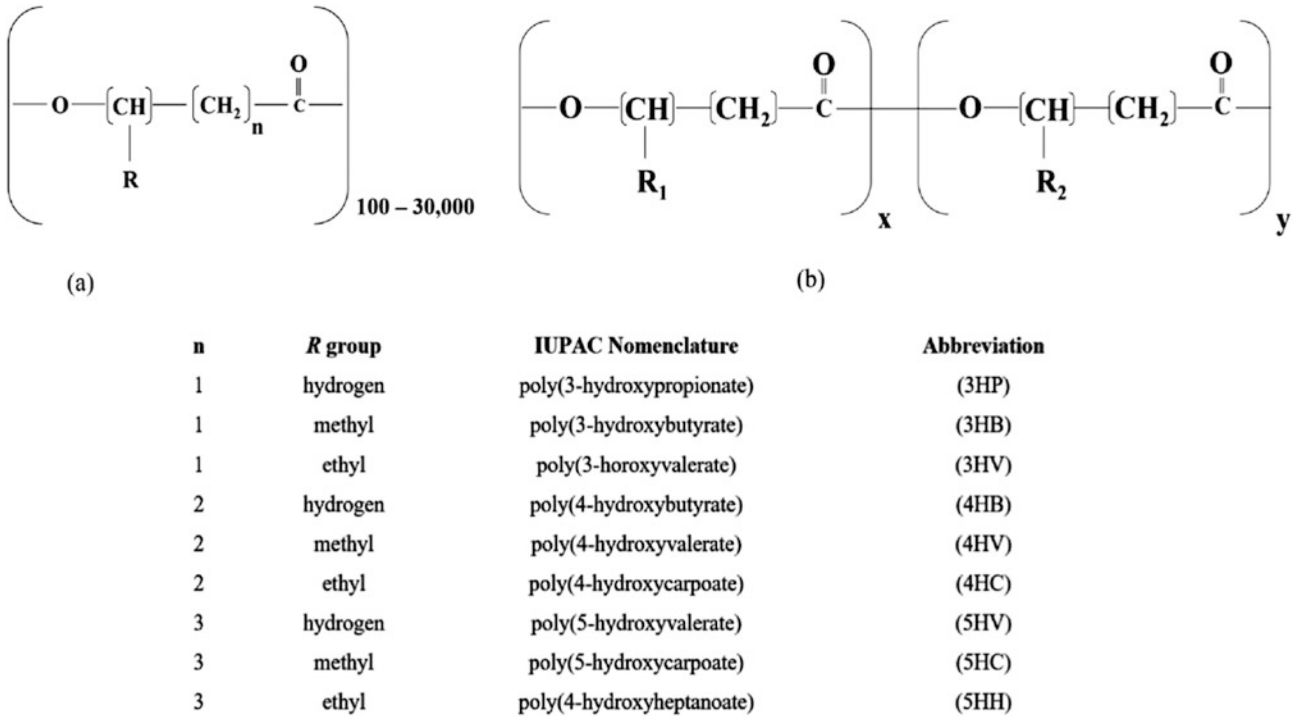
2.5.1 Polyhydroxyalkanoate (PHA)

Polyhydroxyalkanoate (PHA) is a biopolymer that is well-known for its biodegradability, usually present inside microorganisms as a source of energy. PHA obtained from more than 90 genera of both gram-negative and gram-positive bacteria under anaerobic and aerobic conditions. Bacteria can be divided into two groups based on conditions required for PHA production. The first group needs some nutrients such as oxygen, nitrogen, phosphorus, and magnesium for the accumulation of PHA, but this group does not accumulate PHA during the growth phase. The second group does not require any limitation of nutrients and can accumulate PHA during the growth phase (Muhammadi et al. 2015).

Suszkiw (2005) explained polyhydroxyalkanoates are linear and are produced as a by-product by the bacterial fermentation of lipids and sugar. Bacteria produce polyhydroxyalkanoates as the product to store the maximum amount of energy in the form of carbon. On the large scale of the industrial level, the optimum conditions provide the bacteria to obtain the purified polyester by the fermentation of sugar. During the fermentation, more than 150 types of monomers are combined in this polyhydroxyalkanoate production, and these monomers change the whole properties of this family (Fig. 2.7). PHA is a less elastic material and more ductile than plastic material, and it is also biodegradable. Due to the efficiency of these materials, they are more extensively used in the medical industry (Suszkiw 2005).

2.5.2 Polyhydroxybutyrate (PHB)

Polyhydroxybutyrate (PHB) is a macromolecule present inside the bacteria, which acts as inclusion bodies. These are energy reserves that are used by bacteria under unfavorable conditions. They are polymers, and their properties are similar to synthetic plastic like polypropylene. These properties make this polymer a valuable



n	*R* group	IUPAC Nomenclature	Abbreviation
1	hydrogen	poly(3-hydroxypropionate)	(3HP)
1	methyl	poly(3-hydroxybutyrate)	(3HB)
1	ethyl	poly(3-horoxyvalerate)	(3HV)
2	hydrogen	poly(4-hydroxybutyrate)	(4HB)
2	methyl	poly(4-hydroxyvalerate)	(4HV)
2	ethyl	poly(4-hydroxycarpoate)	(4HC)
3	hydrogen	poly(5-hydroxyvalerate)	(5HV)
3	methyl	poly(5-hydroxycarpoate)	(5HC)
3	ethyl	poly(4-hydroxyheptanoate)	(5HH)

Fig. 2.7 Homo- and heteropolymer of polyhydroxyalkanoate (PHA). (**a**) Homopolymer PHA (**b**) Heteropolymer PHA (Mudenur et al. 2019)

product for the synthesis of bioplastic that can replace the petro-based plastic (Kumar and Thakur 2017). A major hurdle in the production of PHB is its high production cost as compared to petro-based plastic. Different techniques such as optimizing fermentation, recovery process, and development of efficient bacterial strain have been used to reduce the production cost of PHB. PHB generally accumulates in the bacteria under a high level of carbon and low levels of nitrogen, oxygen, and phosphorus (Verlinden et al. 2007). The PHB is produced by the activity of acetyl coenzyme-A that is completed in three steps. In the first step, two acetyl-CoAs are converted into acetoacetyl-CoA by the activity of acetyl-CoA acetyltransferase. In the second step, acetoacetyl-CoA is converted into 3-D-hydroxybutyryl-CoA with the help of acetoacetyl-CoA reductase, and in the last step, the final polymerization step is catalyzed by PHB synthase (Peoples and Sinskey 1989).

Suszkiw (2005) stated that the production of the biopolymer poly-3-hydroxybutyrate (PHB) is based on the processing of the bacteria that utilize the raw substrate in the form of glucose, corn starch, or wastewater (Suszkiw 2005). The production of poly-3-hydroxybutyrate by bacteria increases every year, and the properties of the PHB are quite similar to the petro-plastic polypropylene (Gouda et al. 2001). Chen and Patel (2012) also stated that the South American sugar industry is surely going to enhance PHB production at the industrial level. At the primary level, on the industrial scale, isolation of the PHB is based on the physical characteristics, and after that, it can be developed into an apparent film with a high melting point of more than 130 °C (Chen and Patel 2012).

2.6 Bioplastics Obtained from Biotechnological Inventions

Biotechnology plays a significant role in the invention of various methods to get a product in bulk quantity within a short period. By using biotechnology, we can alter the product according to our needs, and product quality can be enhanced. There are a lot of biotechnological methods through which we can obtain our desired product. Biotechnology involves converting plant sugar into plastic, growing plastic in various crops, and producing plastic inside microorganisms. The effort of biotechnology makes bioplastics a more useful and environment-friendly product as compared to conventional plastic (Kumar and Thakur 2017).

2.6.1 Polylactic Acid (PLA)

Polylactic acid is an aliphatic biodegradable polymer that is made from hydroxyl acids mainly polyglycolic acid. Polylactic acid is one of the most important renewable monomers of bioplastic material which are derived from the fermentation of agricultural by-products such as starch-rich substances like sugar or wheat and corn starch. The fermentation process is followed for the conversion of corn and another carbohydrate into the dextrose and finally into the lactic acid (Fig. 2.8). PLA is

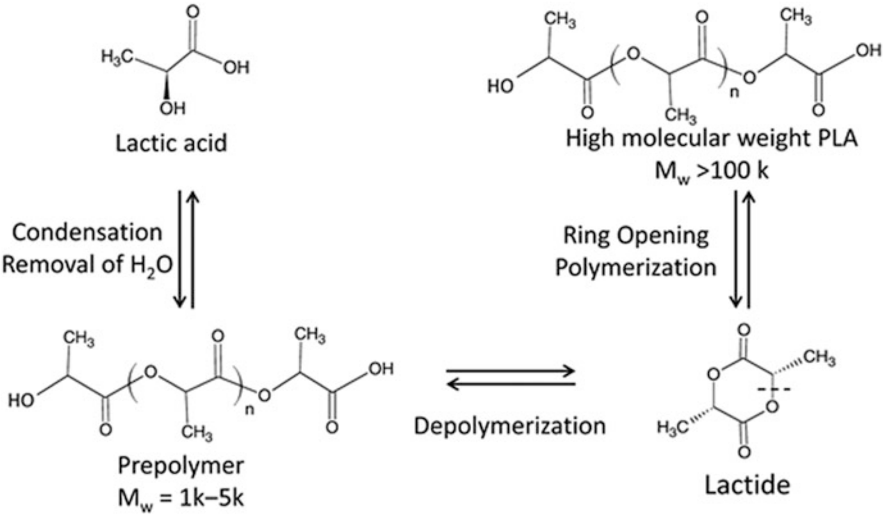


Fig. 2.8 Synthesis of polylactic acid (Ding et al. 2018)

obtained from lactic acid by two different methods. In the first method, the lactic acid cycle produces cyclic lactic acid dimer lactide that further produces PLA with high molar mass. The second method is direct polymerization of lactic acid, usually condensation polymerization, which yields PLA polymer with low molar mass (Polyesters and Albertson 2001). There are two stereo-regular forms of lactic acid: L-lactic acid and D-lactic acid. The lactic acid obtained from petro-chemical is D-lactic acid, while L-lactic acid is obtained from the fermentation of starch. PLA obtained from lactic acid is also called thermoplastic that is a result of biodegradable aliphatic polyester having sufficient potential for the application of packaging (Rhim et al. 2009). PLA pellet formation is directly based on the lactic acid monomers polycondensation or ring-opening polymerization of lactide (Jabeen et al. 2015).

PLA is a packaging material most commonly used for the packing of different materials. The strength of packing material depends upon the ratio of isomers of lactic acid monomers. PLA is the first more safe biobased material that fulfills the requirements of packaging material on a huge scale. Due to its safety and eco-friendly nature, PLA is most actively used in large-scale coatings, film, and injection-molded objects (Rasal et al. 2010). PLA is also modified with low-density polyethylene (LDPE), high-density polyethylene, and polyethylene terephthalate to be used as packaging material. PLA is well-known for its biodegradability in nature due to the presence of ester bonds which act as its backbone. It is much similar to polyolefins and can be converted into plastic by some standard methods such as extrusion and injection molding. PLA is commonly used for packaging purposes as the PLA is present in our body as a nontoxic material. So, when the PLA is used in packaging, food contamination is neglected. By increasing brittleness and thermal properties, PLA requires various modifications such as blending with other polymers and copolymerization. PLA is also copolymerized with polyethylene glycol (PEG) because PEG is well-known for its hydrophilic and biocompatible ability. In this way, PLA improves its hydrophilic property and can be later used in drug delivery systems (Ross et al. 2017).

2.6.2 Polyethylene

Ethylene is the main constituent for the development of polyethylene (Fig. 2.9). The source of ethylene is ethanol, and it has many similar properties. Fermentation is the main process for the synthesis of polyethylene from agricultural feedstocks such as corn or sugar cane. Although bio-derived, polyethylene is nonbiodegradable but has chemical and physical similarities with traditionally synthesized polyethylene. Bio-ethylene is a bioplastic material, and it can also eliminate greenhouse emissions (Shiramizu and Toste 2011).

2.7 Bioplastics Obtained Chemically

The chemical method is one of the most applicable and conventional methods for the synthesis of polymers; there is one suitable method for the production of “bio-polyesters” in a huge quantity. By adopting the chemical method, a large number of “bio-polyester” are produced; however, in the class of “bio-polyester,” polylactic acid (PLA) is one of the materials which can be synthesized commercially in a huge quantity for the production of renewable packaging material (Jamshidian et al. 2010). In the present time, all types of classical packaging materials derived from mineral oil-based renewable resources are derived from the fermentation process.

2.7.1 Polycaprolactones

Polycaprolactone is a crude oil-based bioplastic material that is chemically synthesized by adopting thermoplastic polymer methods. Polycaprolactones contain oil, solvent, water, and chlorine resistance due to these ingredients Polycaprolactones is used as thermoplastic polyurethanes, resins for the synthesis of leather, fabrics, and surface coating due to its adhesive nature (Mousa et al. 2016).

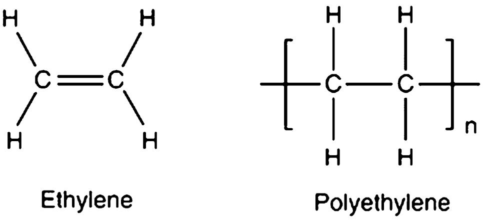


Fig. 2.9 Structure of ethylene and polyethylene (Sherazi 2014)

2.7.2 Polyamides

Aliphatic polyamides, also considered nylons (Fig. 2.10), are among the most significant product polymers. Polyamides also contain amide groups in the backbone of the macromolecule, which makes polyamides heterochain polymers. These are thermoplastic due to wide-ranging presented properties which are actively utilized in the creation of fibers and films, molding compounds (Page 2000). There are three main methods which are used for the synthesis of polyamides: (1) the first method is amino carboxylic acid polycondensation as bifunctional monomers, (2) diamines and dibasic acids both are polycondensed, and (3) the third method is lactams—cyclic amides ring-opening polymerization method and amide monomers contain three to seven ring atoms (Rulkens and Koning 2012). Literature well covers all synthetic pathways as well as polyamide copolymers of various compositions and structures (Hashimoto et al. 2004). Recently, the researches are fully focused on the more significant class of bio-based polyamide thermoplastics, which are partially or entirely manufactured from renewable resources of low cost (Stevens 2013). In the fabrication of bio-based polyamide thermoplastics, castor oil is used as a bio-based monomer and also mass-produced by the fermentation process. The process of synthesis of bio-based polyamides and synthetic polyamide are the same, and there are several profitable yields accessible in the market (Rilsan®11 of Arkema (Colombes, France), Ultramid Balance® of BASF (Ludwigshafen, Germany), Vestamid Terra® of Evonik (Essen, Germany), etc.) (Thielen 2010). The latest research includes the most well-known and fast-developing family of thermoplastic poly-(ester amides) that contain the most effective and valued characteristics of both polyesters and polyamides, i.e., polyesters naturally originated by bio-based castor oil so that is biodegradable and also shows the properties of high tensile strength and high thermal stability (Fonseca et al. 2014). Recently, all consideration is being given to the production of poly-(ester amides) that contain α-amino acids which are actively participated in the biomedical materials (Rodriguez-Galan et al. 2011). The

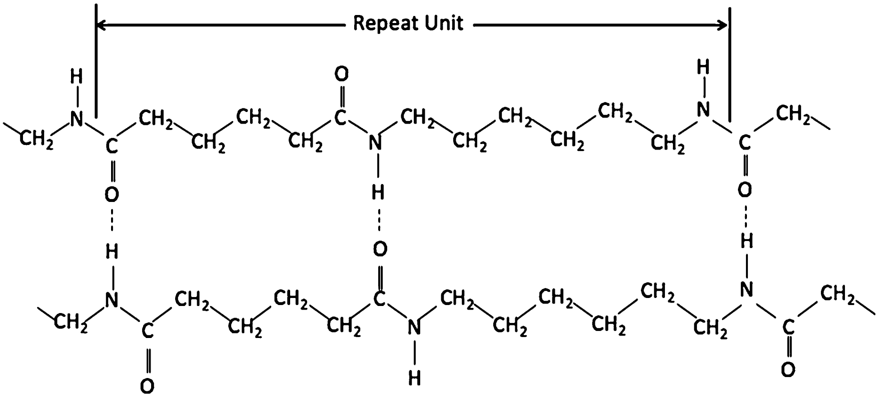


Fig. 2.10 Structure of nylon (Baker 2018)

role of the α-amino acid enhances polymer-cell interactions and also contributes to the addition of functional groups. This whole process is actively involved in the biodegradability of the material.

2.7.2.1 Polyamide (PA11)

Natural oil is the main source of biopolymer polyamide 11 (Fig. 2.11). PA11 is not biodegradable and belongs to a family of technical polymers, and it is also known as tradename Rilsan B and at a commercial scale by the name of Arkema PA11. The properties of PA11 are quite similar to PA12, although, during the production of PA11, emission of greenhouse gases and utilization of nonrenewable assets are depleted. PA11 is superior to PA12 and thermally resistant. Due to extraordinary properties, it is the most commonly used in pneumatic air brake tubing, automotive fuel lines, flexible oil and gas pipes, electric cable anti-termite sheathing, control fluid umbilicals, sports shoes, electric device components, and catheters. PA410 polyamide is another closely related bioplastic material extracted from castor (Levchik et al. 1992).

2.8 Role of Petrochemical Products in the Synthesis of Bioplastics

Petrochemical products played a very effective role in the synthesis of bioplastic materials. Polycaprolactone and polyvinyl alcohol are the main synthetic (petroleum) source product. The origin of polycaprolactone is based on crude oil and chemically synthesized biodegradable thermoplastic polymers. Polycaprolactone shows the properties of the good solvents, and chlorine resistance used in thermoplastic, resin for surface coating, and synthesis of leather and fabrics. Polycaprolactone and polyvinyl alcohol both have limited use, due to low glass transition and melting temperature of 60 °C actively used in the starch-blends.

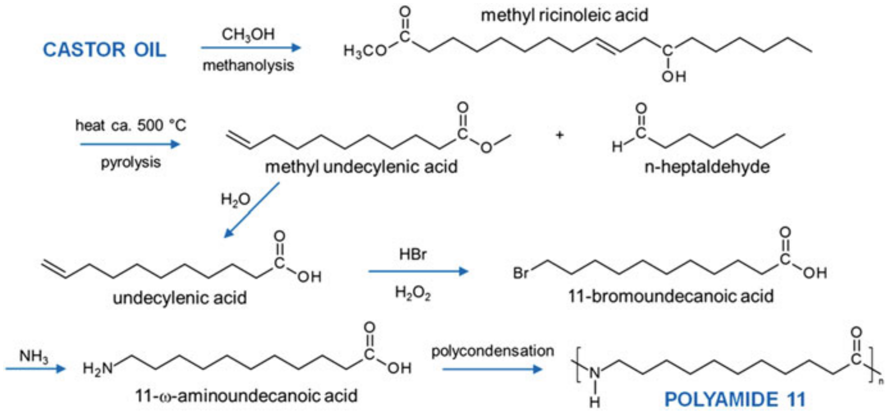


Fig. 2.11 Synthesis of polyamide 11 (Jariyavidyanont et al. 2019)

Polyvinyl alcohol (POVH) is another polymer used in packaging applications. The preparation of POVH is based on the hydrolysis of polyvinyl acetate. The method is based on the starch control over the water solubility, and finally, resin products are formed—although the PVOH biodegradability is still disputed further. Starch and PVOH blends are sensitivite to water, both can be water-soluble (Kumar and Thakur 2017).

2.9 Conclusion and Future Perspective

Production of bioplastic material at the commercial level is costly in the current situation, but the future advancements in the technologies play an effective and viable role in the production of these biomaterials. Researchers are already doing a great job in this field, and some positive results have been obtained. The utilization of bioplastic material in the market is minimal due to rare uses for special purposes such as food items, medical items, and consumable packages. In the future, there will be more possibilities which will eliminate all environmental hurdles we are facing currently due to the petroleum hydrocarbon-derived plastics. To date, the number of polyesters with plastic material is 160, and the number of this bioplastic material increases exponentially by the genetic modification and metabolic genetic engineering techniques. However, some limitation is observed about original microbial strains as compared to the recombinant microbes for the synthesis of novel polyesters. Thus, by utilizing appropriate organisms, many other bioplastic materials could be obtained with different properties and structures. In conclusion, bioplastic materials, due to special characteristics and broad biotechnological application, offer an extremely promising future.

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3

Innovative Technologies Adopted for the Production of Bioplastics at Industrial Level

Ezgi Bezirhan Arikan, Esma Mahfouf Bouchareb, Raouf Bouchareb,
Nevin Yağcı, and Nadir Dizge

Abstract

In the last decade, bioplastic production and utilization have been great attention due to rising environmental problems and consciousness. These materials which can be made from renewable resources or wastes have already been used in a wide range of industrial applications such as packaging, biomedical, agriculture, electrical, structural, and hygiene products. In 2019, approximately 2.1 million tons of bioplastic was produced. Presently, about 50% of bioplastics currently have been created in Asian countries. European countries are the second center of production bioplastics with only a 20% market share. On the other hand, it is expected that this market share will be raised and the global bioplastic production will also be increased by 34% in 2023. However, all these predictions depend on the adaptation and development of bioplastic production technologies at an industrial scale. Although developing countries tend to adopt traditional bioplastic production methods currently in use, innovative technologies direct the bioplastic industries with a competitive approach. Therefore, this study focused on conventional and innovative bioplastic production technologies at the industrial level to support a tactile direction.

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Keywords

Bioplastic · Biopolymer · Innovative technologies · Industrial production

3.1 Introduction

In the last decades, the world has become a planet full of waste and depleted all of its natural resources due to unconscious consumption and rapid population growth (Kakadellis and Harris 2020). Although most countries have developed strategic plans for waste management and natural resources, many have failed in implementation, mostly due to the frenetic use of single-use plastics. So, these versatile materials' global production has reached almost 360 Mtons in 2018 (Plastic Europe 2019). However, they have been at the center of all these environmental problems because of shrinking landfill capacity, low recycling rates, increase of toxic emissions caused by the burning, their nondegradability or durability, and invasion of the oceans by the nano-/microplastics (Arikan and Ozsoy 2015). In light of these increasing environmental concerns, humanity has started to seek new renewable resources, reuse waste, and use more environment-friendly products such as "bioplastics."

In general, bioplastics can be made from renewable resources such as corn and potatoes (Karana 2012) and produce by a range of microorganisms (Luengo et al. 2003). Although its first official discovery dates back to 1926, it is known that the use of bioplastics has increased in the last 20 years (Cooper 2013). European Bioplastics (2019) estimated that 2.43 Mtons of bioplastics would be produced in 2024. Currently, it is known that these innovative materials have been used in many industrial areas such as food packaging (52% in 2019), consumer goods (10%), textiles (10%), agriculture (7%), automotive (7%), coating and adhesives (7%), construction (4%), and other sectors (3%) (Brizga et al. 2020). Although bioplastic is known as one of the most innovative materials of the twenty-first century and has a wide variety of usage areas presently, its production is only about 1% of the annual total traditional plastics manufacturing. It is clear that the bioplastics industry has passed from its infancy to childhood, and all market niches explored. However, to increase global bioplastic production amount and a complete replacement of petrochemical plastics with bioplastics mainly depends on three factors: (1) government policies for sustainable development, (2) production cost, and (3) development of process (bio)-technology (Horvat and Wydra 2017). Whereas there is a growing interest in bioplastics in many countries worldwide, few countries have policies explicitly targeting the bioplastics sector (OECD 2013). On one hand, some countries currently adopt traditional bioplastic production methods in use for entering to market. On the other hand, large blocs such as the United States and Asian countries need innovative technologies to build up bioplastic production capacity. Therefore, this chapter focuses on traditional and innovative production technologies that have shaped the transition from the age of bioplastics. In addition to that, it gives an overview of the development of a strategic direction at the industrial and laboratory scale of bioplastic production.

3.2 Definition of Biopolymers and Bioplastics

Nowadays, there has been a significant shift in the market towards bioplastic from traditional plastic due to its environmental-friendly label. Bioplastic production and consumption will grow more prominent in the future. Because of this, these materials need to be evaluated carefully for sustainability. Otherwise, the use of a product attributed to the environmentally harmless could result in the same damage as traditional plastics. Hence, it is primarily to know their differences from plastics and definitions and classifications of bioplastic. Plastics are defined as long-chain polymers, and they are synthetic/semisynthetic materials (Braun et al. 2005). Although plastics are produced from natural resources such as cellulose, coal, natural gas, and mostly crude oil (Plastic Europe 2020), using additives to improve their performance such as durability make them hazardous for human health and the environment (Lithner et al. 2011). Currently, "bioplastic" is a misused, misunderstood, and abused term in marketing to attract environmentally conscious consumers' attention. Like plastics, they are defined as long-chain biopolymers, but their production processes contain different feedstocks, properties, and applications (Kakadellis and Harris 2020). However, these materials are classified as bio-based and biodegradable. Bio-based bioplastics initially refer to producing from biological material. They are made from partially or fully renewable resources and classified as first-, second-, and third-generation bioplastics according to the utilization of renewable resources (Brodin et al. 2017). First-generation bio-based bioplastics are produced from plants and crops consumed by humans and animals such as corn, wheat, sugarcane, potato, sugar beet, and rice. Due to concerns about on land-use, competition for arable lands, and hunger in the world (Nanda et al. 2015), debate on the use of plants in the production of bio-based bioplastics has led to arise second-generation bio-based bioplastics. These types of bioplastic can produce from cellulosic materials such as bagasse, wheat straw, pulp, corncob, palm fruit bunches, and grass or waste of raw materials used in first-generation ones such as vegetable oil or wastewater, which is not suitable for human or animal nutrition (Brodin et al. 2017). For example, polylactic acid (PLA) is a first-generation bio-based bioplastic due to corn production. On the other hand, Bio-polyethylene (Bio-PE) is the second generation due to the utilization of sugarcane of waste fats and oils on production. Finally, third-generation bio-based bioplastic refers to produce from biomass such as algae (Barrett 2018a, b). Due to these aspects, bio-based bioplastics have been addressed to the bioeconomy of the future (Brodin et al. 2017).

Biodegradable bioplastics are fully degraded by microorganisms without leaving visible toxic remainders (El-Kadi 2010). In other words, they can be degraded completely by a microbial attack in a short period, under appropriate environmental conditions (Albuquerque and Malafaia 2018). European Bioplastics (2016) defines it as degraded by naturally occurring microorganisms into the water, carbon dioxide,

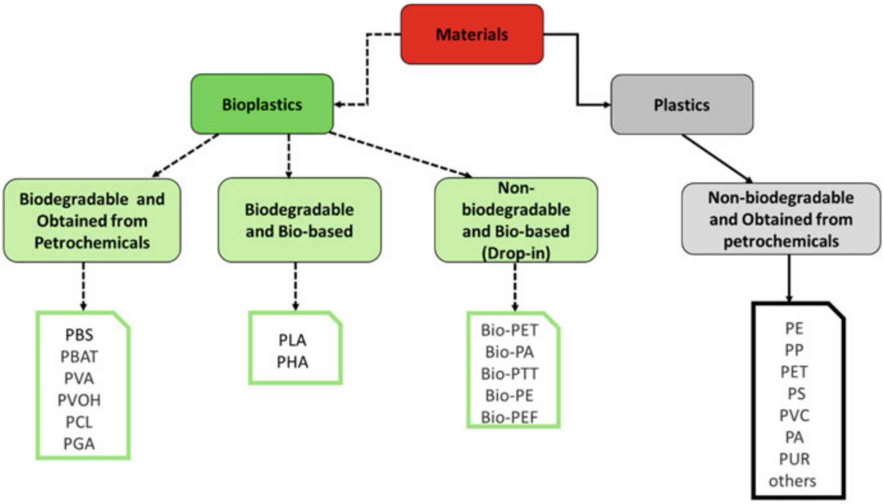

Fig. 3.1 Classification of plastics and bioplastics

methane, and inorganic compounds under certain conditions. It means biodegradable bioplastics could not be degraded in the backyard because of biotic and abiotic factors. Another critical point to be known is that bio-based bioplastics are not always biodegradable (e.g., PLA and Bio-PE) (Polman et al. 2020), and these nonbiodegradable bioplastics called drop-in bioplastics recently (Brizga et al. 2020; Hwang et al. 2020). On the other hand, plastics obtained from petrochemical resources such as polycaprolactone (PCL), polyglycolic acid (PGA), and polybutylene succinate-co-adipate (PBSA) are classified as biodegradable. At this point, it is essential to understand that conventional plastics (polymers, obtained from petrochemical resources) and bioplastics (biopolymers, obtained from renewable resources or petrochemicals) (Zhong et al. 2020) are (1) biodegradable or not and (2) biobased or not (Hwang et al. 2020). Figure 3.1 provides a visual distinction according to these definitions.

3.3 Recent Developments in the Bioplastic Industry

Although the term of bioplastic has been known in recent years, its development dates back to the nineteenth century. American inventor John Wesley Hyatt Jr. patented the cellulose-based coating instead of the ivory used in the production of billiard balls in 1869 (El-Kadi 2010). Cigarettes accidentally fired the balls, and this attempt was prevented. However, Hyatt continued to work on his project and developed celluloid, mostly known as film tape today (El-Kadi 2010). In 1897, Wilhelm Krische and Adolf Spitteler produced a plastic made from casein in milk marketed as “Galalith.” This type of plastic was used in materials such as jewelry and electronic equipment in these years. However, milk became a raw material in short supply, and the plastic produced from casein lost its importance due to the abundance of crude oil after 1945 (Arikan and Ozsoy 2014).

In the 1920s, Henry Ford tried to use soybeans in automobile production. Ford was motivated by the desire to find nonfood applications for agricultural production surplus. Finally, Ford produced a car prototype made from soybeans, and it was exhibited in 1941 with a big advertisement. However, at the end of the year, a plastic car's interest decreased due to war and other reasons (El-Kadi 2010). In 1926, French chemist and bacteriologist Maurice Lemoigne discovered the polyhydroxybutyrate (PHB), an intracellular biopolymer produced from the Gram-positive bacterium *Bacillus megaterium* (Anderson and Dawes 1990). Nowadays, it is known that these biopolymers have been used in a vast industrial application such as razor blades, kitchenware, diapers, pens, combs, gloves, bags, cosmetic product containers, packaging materials such as shampoo bottles, glasses, and milk boxes (Anjum et al. 2016) In 1932, Poly(lactic acid) or polylactide (PLA), which is the most broadly used biodegradable bioplastic type, was developed by Carothers (at DuPont) (Farah et al. 2016). In the 1950s to 1960s, industrial-scale production of PHBs from microorganisms was evaluated in the United States, but this attempt was unsuccessful due to the fall in oil prices (Barrett 2018a, b). Finally, a company named Imperial Chemical Industries established in the United Kingdom in 1983, succeeded in producing the Biopol that is a type of bioplastic produced from microorganisms on a commercial scale (Barrett 2018a, b). Later, Novamont (in 1990), Cargill and Dow Chemicals (in 1997), Metabolix (in 2001), and many biopolymer manufacturers entered the industry. All historical development of bioplastics is illustrated in Fig. 3.2.

From a broader perspective, it is thought that plant-based resources such as carob and cotton waste for plastic production triggered the discovery of bioplastics (Stevens 2002). With the discovery of oil in the early 1900s, bioplastics remained in the background, and synthetic plastics began to be used in many areas (El-Kadi 2010). However, bioplastics have attracted again due to the oil crisis in the 1970s. Due to increasing environmental damage and the global awareness of society, interest in bioplastics has grown with the support of ecological campaigns after the 2000s (Albuquerque and Malafaia 2018). According to European Bioplastics (2019), global bioplastic production was 700,000 tons in 2010 (Soroudi and Jakubowicz 2013), 1657 Mtons in 2014, 2054 Mtons in 2017, and 2114 Mtons in 2019. Furthermore, it is expected that global production capacity will increase by approximately 2.43 Mtons in 2024 (European Bioplastics 2019). Currently, 45% of bioplastics are produced in Asian countries. European countries are the second center of production bioplastics with a 25% market share. On the other hand, North and South America participated in the global bioplastics market with 18% and 12%, respectively (European Bioplastics 2019).

Presently, biodegradable bioplastics such as polybutylene adipate terephthalate (PBAT), polybutylene succinate (PBS), polyhydroxyalkanoate (PHA), PLA, and starch-blends are the most produced bioplastic type with 55.5% (1.16 Mtons) of the total bioplastic market (Brizga et al. 2020). Among these bioplastics, PHAs are the promising bioplastic type due to their remarkable growth rates (European Bioplastics

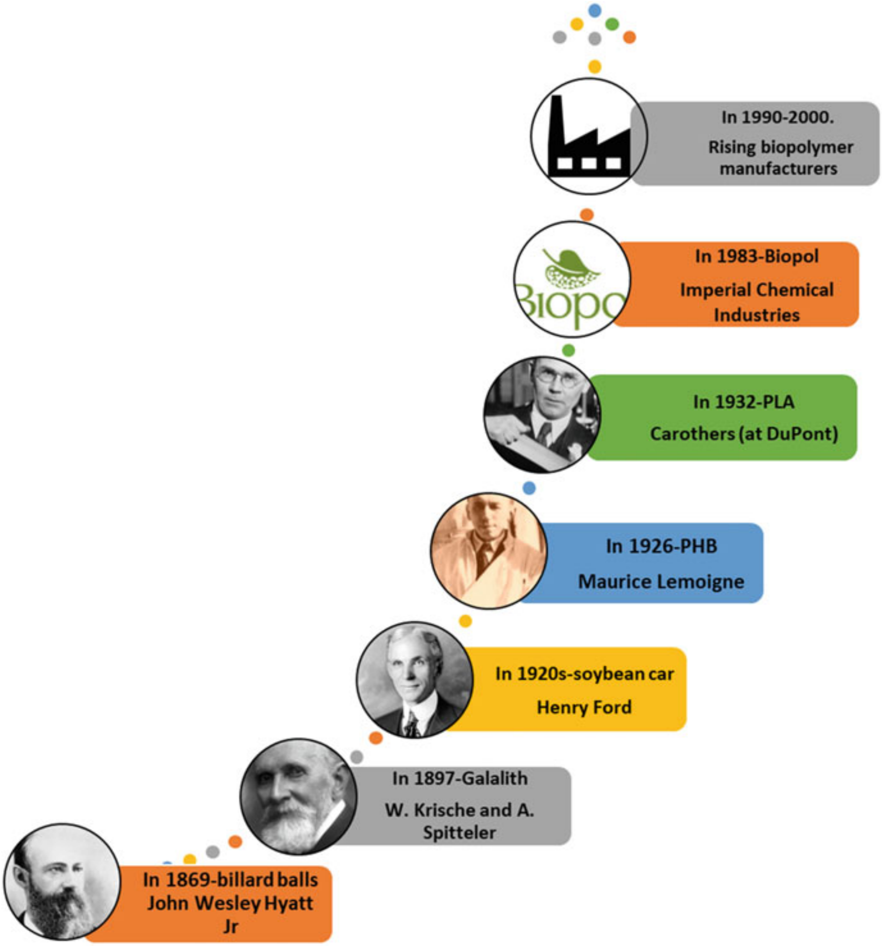

Fig. 3.2 Historical development of bioplastics

2019). On the other hand, the market share rate of biobased/nonbiodegradable bioplastics called drop-in is 44.5% of the total bioplastic market (European Bioplastics 2019). Among them, bio-PE (polyethene), bio-PET (polyethylene terephthalate), and bio-PA (polyamides) are the most popular bioplastics produced in 2019 (Brizga et al. 2020). It is also expected that polyethylene furanoate (PEF), which is defined as a 100% bio-based alternative to petro-based PET (Hwang et al. 2020), will enter the bioplastics industry in 2023 (European Bioplastics 2019). Nowadays, bioplastics are used in many industrial areas, including textiles, automotive, and cosmetics, but still, the largest field of application is packaging.

3.4 PHA Production

Among the bioplastics, polyhydroxyalkanoates (PHAs), a family of biopolymers, have attracted attention due to their degradability, compostability, biocompatible, and nontoxic features (Tarrahi et al. 2020). Polyhydroxybutyrate (PHB) is the most recognized and researched type of PHA currently (Sabbagh and Muhamad 2017). However, many biopolymers are belonging to this family, such as poly (3-hydroxypropionate), poly(3-hydroxyhexanoate), poly(3-hydroxyoctanoate), and poly(5-hydroxyvalerate) (Rajan et al. 2018). Various microorganisms can synthesize PHA as a storage material in the presence of excess carbon sources and under limited nutritional conditions (nitrogen or phosphorus) (Lee et al. 1999; Anjum et al. 2016). It is known that PHAs have been synthesized by more than 300 microorganisms including bacteria such as *Wautersia eutropha*, *Cupriavidus necator*, *Thermus thermophilus*, *Hydrogenophaga pseudoflava*, *Saccharophagus degradans*, *Azohydromonas lata*, *Rhodobacter sphaeroides*, and *Zobellella denitrificans* (Reddy et al. 2003) and algae such as *Nostoc muscorum*, *Spirulina platensis*, *Synechococcus elongates*, *Aulosira fertilissima*, *Botryococcus braunii*, and *Dunaliella salina* (Costa et al. 2019). As demonstrated in Fig. 3.3, PHA production consists of five significant steps: fermentation, harvesting, pretreatment, extracting PHA from microorganisms, and purification.

PHA production mainly starts with fermentation after microorganism inoculation onto a substrate such as corn, wheat, and rice (Fig. 3.3). During fermentation, microorganisms accumulate PHA biopolymers as storage compounds for energy and carbon in their cell under special fermentation conditions. At the end of the fermentation, harvesting or separating of biomass (cell) is conducted with centrifugation, sedimentation, or filtration (Pérez-Rivero et al. 2019). Following harvesting, PHA granules covered by enzymes and structural proteins in the cell extracted from the biomass (Koller 2018). The extraction step includes different methods such as chemical (chloroform/methanol, sodium hypochlorite, methylene chloride, sodium dodecyl sulfate, chloropropane, and 1,2-dichloroethane), mechanical (bead milling and high-pressure homogenization, ultrasonication), and biological (freeze-drying) (Pérez-Rivero et al. 2019). However, combined methods are mostly applied to reducing the amount, costs, and harmful effects of chemicals (Murugan et al. 2016). At the end of the last step, extracted PHA is purified by washing, grinding, and polishing according to the final product's final requirements (Kunasundari and Sudesh 2011).

Production of PHA at an industrial scale has been focusing on using the first-generation plant and bacteria mostly. Some companies producing bacterial PHA and their production costs are shown in Table 3.1. PHA cost ranges from 1.5 to 5.0 €/kg, whereas the production cost of petrochemical plastic such as PE and PP is about 1.12–1.6 €/kg (Kourmentza et al. 2017). Although PHAs are one of the most promising bioplastics, they have not been used more in the industry due to their high cost (Amaro et al. 2019). It is known that the cost of PHA effects by three main factors, including the cost of carbon substrate, the extraction methods, and sterilization steps (Dias et al. 2006; Ivanov et al. 2015; Kourmentza et al. 2017). However,

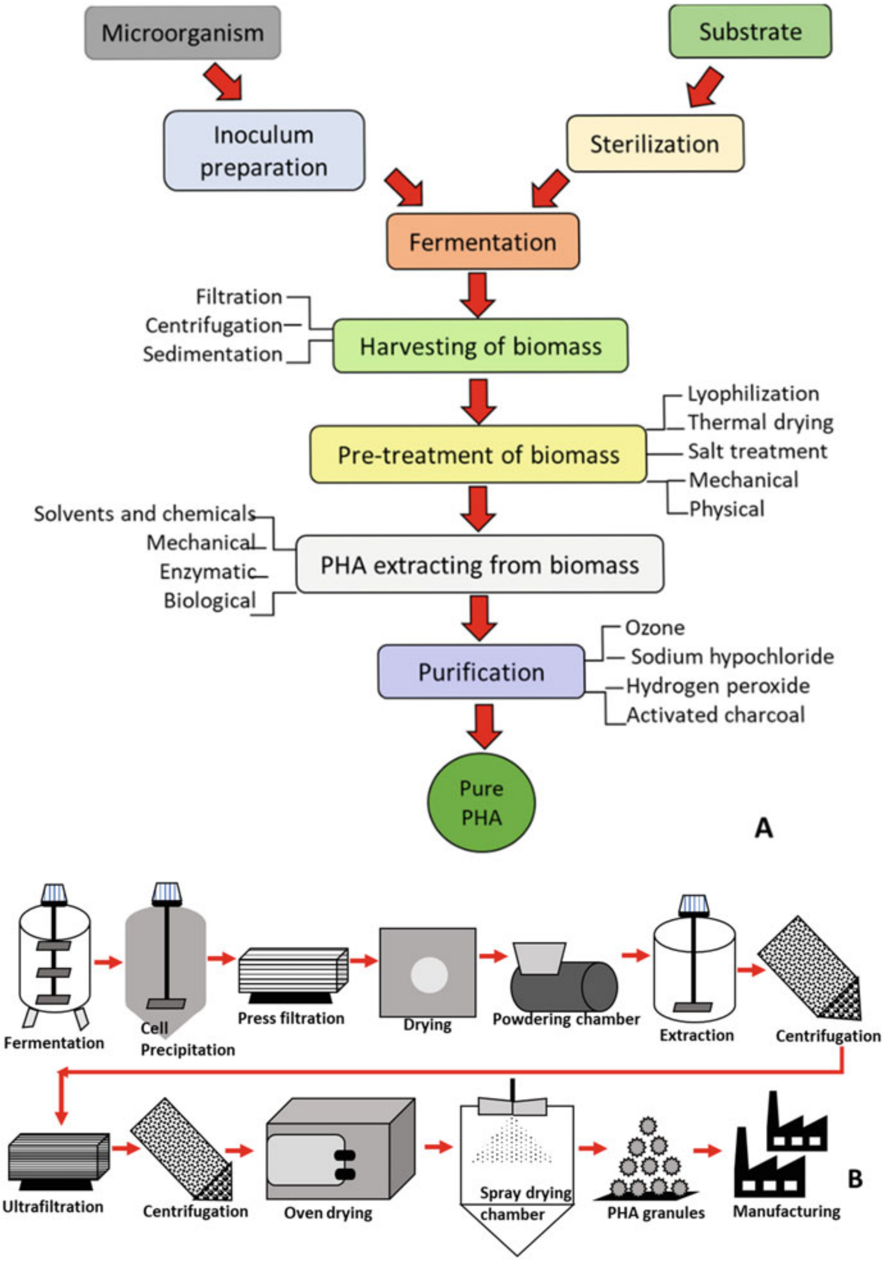

Fig. 3.3 (**a**) Basic production steps of PHA. (Adapted from Kreyenschulte et al. 2014, Pérez-Rivero et al. 2019) (**b**) Industrial production steps of PHA

Table 3.1 PHA production cost by some companies (Kumar et al. 2020)

Company Name	Country	PHA	Microorganism	Price (€/kg)
Biomer Inc.	Germany	PHBV and PHB Biomer®	*Alcaligenes latus*	3.00–5.00
Bio-On Srl.	Italy	PHB, PHBV minerv®-PHA	*Cupriavidus necator*	–
Biomatera	Canada	PHA Biomatera	Non-pathogenic, non-transgenic bacteria	–
PHB Industrial SA.	Brazil	PHB, PHBV (BIOCYCLE®)	*Alcaligenes* sp.	–
Tianan Biologic, Ningbo	China	PHBV, PHBV + Ecoflex blend Enmat®	*Ralstonia Entropha*	3.26
Telles	US	PHB Mirel™	*Ralstonia eutropha*	1.50
Mitsubishi Gas Chemical Company Inc.	Japan	Biogreen®, PHB	–	2.50–3.00
Lianyi Biotech	China	PHBH Nodax™	–	3.70

pure sugars such as glucose or sucrose or other sugar-based compounds such as corn, which lead to high cost, were used widely for PHA production (Serafim et al. 2008). Thus, recent studies have focused on using low-cost carbon substrates such as waste or wastewater to simultaneously reduce PHA production and disposable waste costs (Nielsen et al. 2017). In this concept, a lot of low-cost carbon sources such as lignocellulosic raw materials (wood, xylose, hemicellulose hydrolysates, wheat bran, etc.) (Tamer et al. 1998), whey (hydrolyzed soy and malt, hydrolyzed whey and whey molasses) (Koller et al. 2008), molasses (sugarbeet, cane-sugar, and soy molasses) (Verma et al. 2003), waste cooking oils (olive oil, coconut oil, soybean oil, palm oil, etc.) (Solaiman et al. 2006), and wastewater (brewery, palm oil, paper, food) were studied (Adeleye et al. 2020). For example, Santimano et al. (2009) investigated the utilization of molasses for PHA production, and they reported an increase in PHA production to 6.0 g/L. Moreover, besides laboratory and pilot-scale studies, some companies have started to produce PHA from waste materials on an industrial scale. For example, PHAs are produced from molasses as agricultural waste material by Bio-on, a firm based in Italy (Bio-on 2016). Another company, the government-owned SIRIM Bioplastics from Malaysia, used palm oil mill effluent (POME) and crude palm kernel oil to produce PHA at a pilot scale (SIRIM 2020).

From the biotechnological point of view, another cost-effective solution is using mixed microbial consortia (MMC) for PHA production due to eliminating the sterilization step (Serafim et al. 2008). MMC can be defined as a microbial population of unknown composition capable of performing specific intra- and extracellular reactions. Therefore, activated sludges in wastewater treatment plants are considered MMC. As illustrated in Fig. 3.4, the PHA production process using mixed microbial cultures is usually carried out in a mostly sequence of stages depending on the

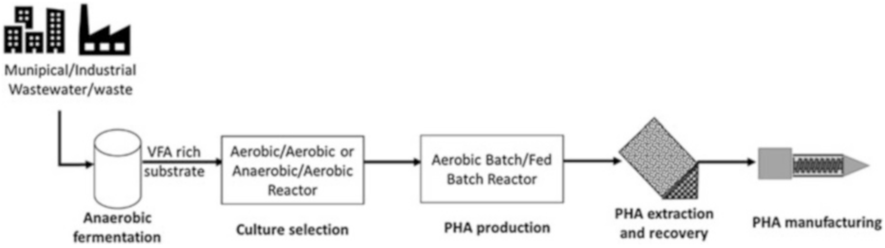

Fig. 3.4 PHA production process of waste/wastewaters

substrate type (Serafim et al. 2008). The first step is acidogenic (anaerobic) fermentation for the production of volatile fatty acids (lactic acid, acetic acid, propionic acid) from carbon-rich wastewaters. Following VFA production, culture selection (enrichment) was carried out mostly in aerobic dynamic feeding conditions in a sequential batch reactor to produce biomass with PHA accumulation potential (Bengtsson et al. 2017). Another stage is the production (accumulation) of PHA in the biomass to maximize the biomass PHA content (Pittmann and Steinmetz 2017). Then, the PHA produced in the accumulation step is extracted and purified at the last stage (Albuquerque et al. 2007). This production process can be applied to complex substrates such as olive mill wastewater cheese whey and other food wastes to obtain a more homogeneous PHA production (Kourmentza et al. 2017).

3.5 Manufacturing Methods of Bioplastics

Bio-based bioplastics are produced from natural or renewable sources after a series of stages as illustrated in Fig. 3.5 (Syed 2016; Tonuk 2016). Raw materials as sugarcane, corn, and potatoes are collected from their fields and processed to extract their starches (Bastioli et al. 2013; Tsang et al. 2019). The starches are treated further in refineries by using fermentation or special enzymes to synthesize the chemical constituents that react to make bioplastic. The compounds can be refined to fit manufacturers' recommendations for different products (Mostafa et al. 2018; Tsang et al. 2019). Then bioplastics producers utilize granules or pellets of the compounds to make plates, utensils, cups, carpeting, and many other products (BMEL 2016). The advantage of most bioplastics, biodegradable ones, is that they can be disposed of with biodegradable wastes. The organic waste shall compost and return to the ground as mulch to support new crops and complete the cycle (Syed 2016).

Bioplastics are like any other regular polymer or plastic. It can be processed using traditional techniques, for instance, injection molding and compression molding. However, the way of bioplastics synthesis does not affect biodegradability (Srikanth 2011; Thielen 2014). Procedures can be divided into two categories, synthetic or biotechnological, where most plastics preparation is based on a natural polymer that is chemically or mechanically treated (Syed 2016). Particular attention should be

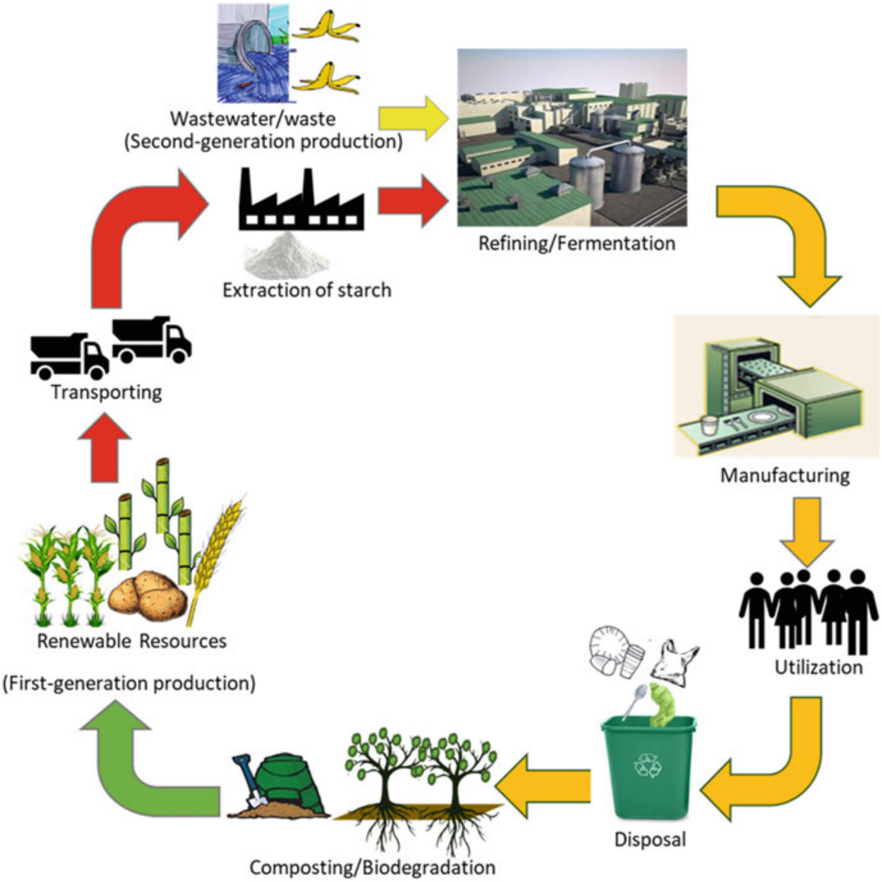

Fig. 3.5 The life cycle of bioplastics

given to the bioplastic itself rather than its processing. For example, to avoid hydrolytic degradation of bioplastic, moisture needs to be removed from the polymer before processing. Plastics would be dried for a determined period for moisture removal. Additionally, the processing should be achieved under known monitored humidity (BMEL 2016; Syed 2016; Thielen 2014).

Most biodegradable plastics are produced by ring-opening polymerization reactions (BMEL 2016). In the injection molding and extrusion process, too high processing temperatures might result in monomer formation because of the thermodynamic equilibrium between the reverse and the forward reaction. This additional monomer can perform as a plasticizer distressing material mechanical properties and degradation kinetics. Consequently, these materials are required to be processed at low temperatures. Bioplastics are remarkably different from conventional plastics in material features. The distinctive fine processing window makes them a challenge to process. This leads to the processing of equipment adjustment (Syed 2016). Most bioplastics can be manufactured using an assortment of processing methods (BMEL 2016; Syed 2016; Thielen 2014). Examples of processing methods are listed as injection molding, compression molding, film casting, blow molding, blown film extrusion, thermoforming, and fiber spinning.

3.6 Traditional Technologies for the Manufacturing of Bioplastics

The most common traditional technologies for bioplastics production to be discussed in this section are injection molding and compression molding processes.

3.6.1 Injection Molding

Injection molding is the most commonly used process for thermoplastic production, particularly for those with a complex shape, and high dimensional accuracy is necessary (BMEL 2016; Syed 2016). Bioplastics processes like traditional thermoplastics as polycarbonate (PC) and acrylonitrile butadiene styrene (ABS) can be processed in a conventional machine, as illustrated in Fig. 3.6. Though bioplastics are semicrystalline and have low melting temperatures, they are relatively slow to crystallize (Bastioli et al. 2013). The improvement of cycle time and heat resistance can be achieved by utilizing nucleation technology (Syed 2016). Bioplastics are likely to stick to metal surfaces while processing and absorb moisture on contact with ambient air leading to dilution of plastic drying (Syed 2016). Bioplastics are hydroscopic and should be dried to avoid a decrease in melt viscosity and molecular weight, in addition to enhanced potential for flashing and brittle parts (BMEL 2016). Excessive drying temperature results in soft and agglomerate material in the drying hopper. However, at low drying temperatures, the material will not dry as readily. To keep the temperature in the required range, it is suggested to utilize

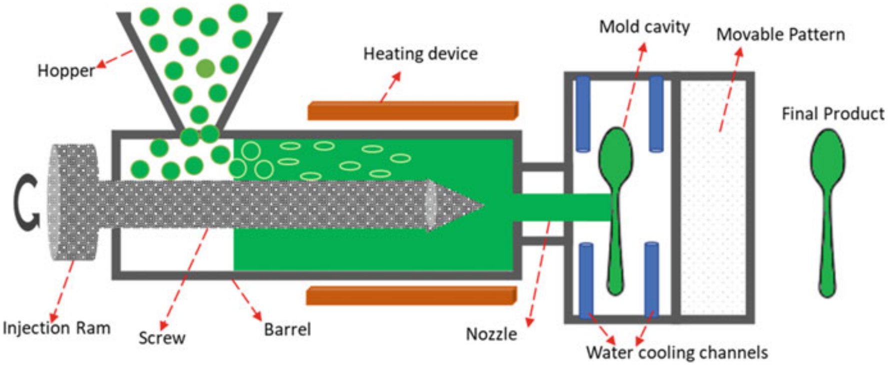

Fig. 3.6 Injection molding machine scheme

fluid-bed crystallizers, hopper agitation, infrared crystallizing, and drying units (Srikanth 2011; Syed 2016).

As a good practice, after using an injection molding machine, it is recommended to purge the bioplastics from the machine. It is known that it is challenging to process bioplastics between the point of decomposition and melting point. If the polymer is exposed to excessive heat, gels, black specs, and yellowing are formed during the production process. The mentioned materials are hygroscopic and sensitive to moisture. Therefore, the following parameters must be monitored closely during processing: screw speed, melt temperature, proper drying, and injection speed (Syed 2016).

3.6.2 Compression Molding

The compression molding process is characterized by two matched metal molds used to manufacture a composite product. The compression molding machine is composed of a stationary plate and a movable plate, as shown in Fig. 3.7. Matrix and reinforcement are positioned in the metallic mold, and the entire assembly is kept in between the compression molding machine. The matrix could be a biodegradable polymer, while reinforcement is fiber or support material. The two parts of the compression molder are brought together with the application of heat and pressure. The polymer takes the shape of the cavity and the shape of the mold. A remedial step of the composite may be necessary either at ambient temperature or at a relatively

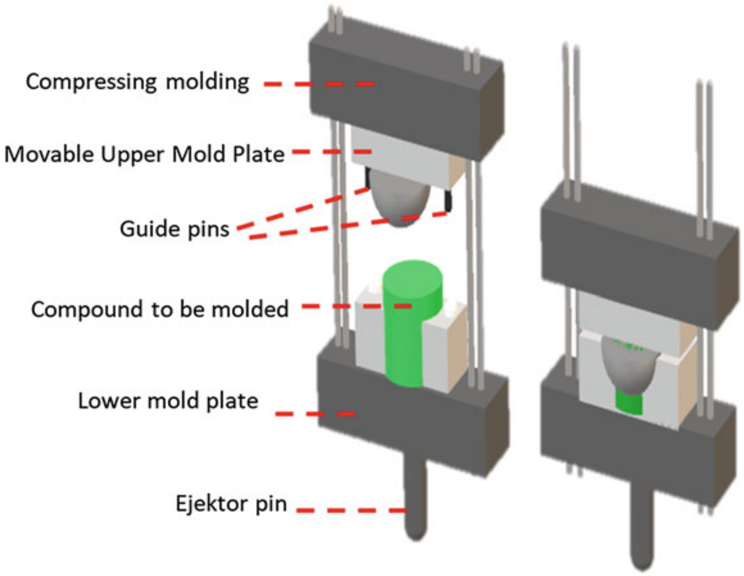

Fig. 3.7 Schematic compression molding machine. (Adapted from Syed 2016)

higher temperature. The mold is then opened, and the product is removed for the next operations (BMEL 2016; Syed 2016).

Three main parameters are considered in compression molding technology to obtain the desired properties of the composite product. Pressure, temperature, and time are essential and need to be optimized (Syed 2016). The insufficient applied pressure may lead to poor interfacial adhesion of fiber and matrix (BMEL 2016). More pressure is applied to cause fiber breakage and expulsion of resin from the composite system. At low temperatures and in particular for thermoplastics, the viscosity of polymer increases, and therefore, fibers cannot get correctly wetted. If the temperature is much higher than desired, fiber characteristics may get changed. If time is too low or too high, the material may have defected. Besides these three main parameters, other processing factors may affect the production process such as plates closing rate, mold wall heating, and de-molding time (Syed 2016).

3.7 Innovative Technologies for the Production of PHA

Recent developments on bioplastic showed that the development stage is classified as an infancy period and childhood period until today. Briefly, most bioplastics producers were focused on only producing and marketing from first-generation plants at the infancy period. Besides, most producers used petrochemical/bioplastic blends for commercial production due to a lack of legislation and classification. However, it is realized that these blends were harmful to the environment because of labeling "biodegradable." In the childhood period, because of rising concern, standardization and legislation, including classification and definition, were published by some organizations such as International Organization for Standardization (ISO), European Standards (EN), and the American Society for Testing and Materials (ASTM). To reach adulthood, it is necessary to apply innovative approaches compatible with technology. With this concept, innovation approaches are summarized in the following subsections.

3.7.1 Waste Utilization/Valorization

In recent years, wastes have gained importance due to obtaining new valuable products as an alternative option for disposal and landfilling (Kanani et al. 2020). Therefore, wastes are considered as unwanted and worthless material in the new sustainable world. On the other hand, as mentioned before, the use of first-generation resources such as sugar in bioplastics production causes high production costs. Consequently, various studies showed the potential utilization of waste oils, industrial by-products, agricultural feedstocks, and wastewater for bioplastic production. For example, WHEYPOL Projects funded by the European Union reported that the cost of PHA production was below 3 €/kg when whey from the dairy and cheese-making industry was used as a carbon source (Kumar et al. 2020). In this concept, waste valorization for bioplastic production could solve the high production cost of bioplastics simultaneously with waste disposal. However, these cost-effective substrates should meet some basic requirements for biotechnological production of bioplastic as mentioned below (Koller and Braunegg 2018):

1. Waste should be sufficient and consistently available.
2. Its composition should be stable and resistant to microbial degradation.
3. If the microbial degradation rate of waste is high, it should be storable.
4. It should be easy to handle and transport.

Although the production of bioplastics from waste has many advantages, the final bioplastic product may contain toxic substances, which prevents its usage in some industrial areas such as cosmetics (Kumar et al. 2020). Hence, the process cost used to remove undesirable substances/components from the final product is one of the points to consider.

3.7.2 Engineered Microorganism and PHAome

In some cases, large-scale production and commercial applications of bioplastics could be limited due to complicated production processes (Tsang et al. 2019). Production processes of PHAs from wild-type microorganisms involve a complicated extraction process for the recovery of PHA (Reddy et al. 2012). Moreover, production from wild-type microorganisms does not provide a high yield of production. For this reason, recent research has been focused on the production of PHA from genetically engineered microorganisms. Genetically engineered bacteria can provide accelerated growth, high cell density, simplification separation, and reduction in bio-production cost (Jiang and Chen 2016). It is known that PHAs can synthesize diverse molecular weights and various monomer arrangements and ratios by the bacteria (Chen and Hajnal 2015). Genetically engineered bacteria can synthesize single selective monomers instead of a mixture of copolymers (Kumar et al. 2020). It is also called the “PHAome concept.” This concept provides producing defined PHA structures and constant molecular weights from bacteria (Chen and Hajnal 2015). Recent advances in engineered microorganisms improve PHA biosynthesis, including ribosome-binding site (RBS) optimization, promoter engineering, chromosomal integration, cell morphology engineering, and cell growth behavior reprogramming (Zhang et al. 2020). When considering PHA production from “waste” and “engineered microorganisms” together, the most innovative approach considered as combining these two production processes as a techno-economic way.

3.7.3 Recycling and Symbiotic Technologies

Circular bioeconomy (CB) mainly targets these four principal “actions”: reduction, reuse, recycle, and return to nature. Therefore, circular bioeconomy is based on

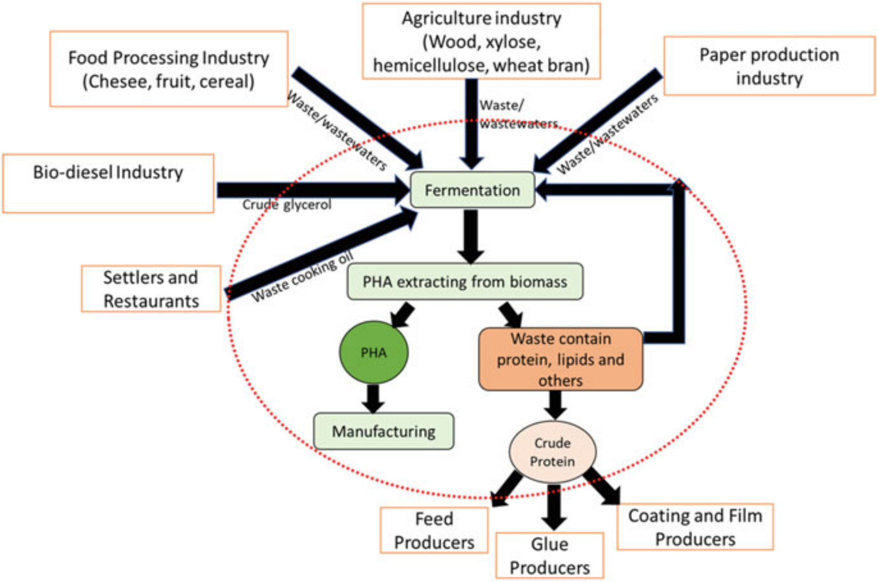

Fig. 3.8 A symbiotic approach way for the PHA production

developing a waste stream recycling for a sustainable future. In this concept, CB recommends not only the production of PHA from waste or by-products but also the use of waste generated in the PHA production process. It is known that bacterial cell contains proteins about 50% of its dry weight (Xiao et al. 2017). Therefore, after the extraction stage in the production of PHA from bacteria, recovery of protein, lipids, polysaccharides, and nucleic acids from cells is considered as another bioeconomic solution (Kumar and Kim 2018). After the characterization analysis, the recovered protein could be used as animal feed, pet food, glue, or a bioplastic component (Yadav et al. 2020). The refeeding liquid, which contains protein to the fermentation stage as a nutrient source, could be another bioeconomic solution for PHA production (Koller 2015). In this context, the PHA production industry evaluated with a symbiotic approach, as shown in Fig. 3.8.

3.8 Conclusions

PHA production is one of the key elements for developing the bioplastic industry. The bioplastic industry's history, classifications, production, and manufacturing methods are elaborately discussed in the chapter. It is to be emphasized that the advancement of PHA technology depends on innovative approaches such as waste valorization, engineered microorganisms, and symbiotic technologies in a greener future. Further research should include the innovative production of PHAs compatible with bioeconomy and environmental sustainability.

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Processing of Commercially Available Bioplastics

4

Raluca Nicoleta Darie-Niță and Maria Râpă

Abstract

Due to the environmental concerns related to the use of nondegradable polymers, the market for bioplastic-based materials is increasing. Currently, bioplastic materials are used in bags, medical devices, food packaging, automotive, and electronics areas. Not all bioplastics are biodegradable, but they are derived from renewable resources and permit the modification of their properties by adding suitable additives in order to have good mechanical and thermal properties as well as easy processing at an industrial scale. Polyesters like poly(lactic acid) (PLA), polybutylene adipate terephthalate (PBAT), and polyhydroxyalkanoates (PHA), together with starch-based materials are the most representative commercial bioplastics available on market. This chapter reports the main manufacturers of bioplastics and the traditional and emergent technologies for their processing to commercially use, highlighting the processing parameters and the main advantages/disadvantages of each technology.

Keywords

Bioplastics · Extrusion technology · Injection molding technology · Thermoforming · Electrospinning · 3D printing · Market

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4.1 Introduction

Bioplastics refer to bio-based and/or biodegradable plastics which have similar properties as conventional plastics but correspond well to environmental strategy and market demand (Fahim et al. 2019). Although bioplastics represent 1% of 320 million tons of plastic produced annually (Fahim et al. 2019; Liliani et al. 2020), their demand for the market is increased. The global price for commercial bioplastics was valued to be over $17 billion USD in 2017, and it is estimated to reach $43 billion USD by 2022 (Price et al. 2020). Among bio-based plastics, the poly(lactic acid) (PLA) is one of the commercially available biopolymers derived from natural resources such as corn starch and sugar cane. PLA possesses excellent film-forming nature and barrier properties against water, air, and oil-grease (Sundar et al. 2020) and shows biocompatibility, and its properties are comparable with those of traditional polymers such as poly(propylene) (PP), poly(styrene) (PS), and poly (ethylene) (PE). In addition, PLA is more sustainable than petroleum-derived plastics (Labonté and Dubois 2015) and biodegradable under industrial composting conditions (Musioł et al. 2016). Poly(butylene adipate-co-butylene terephthalate) (PBAT) is a fully biodegradable, flexible aliphatic-aromatic copolyester designed to improve the toughness of PLA blends (Chiu et al. 2013).

Polyhydroxyalkanoates (PHAs) produced through biotechnology are the competitors for PLA, being commercially available for medicine, agriculture, construction, and packaging applications (Adeleye et al. 2020). PHA can be processed by injection molding, extrusion, and extrusion bubbles into films and hollow bodies (Bugnicourt et al. 2014). The main advantage of PHA blends is that they are biodegradable in a wide range of environmental conditions (Cinelli et al. 2019). The high price of PHA ranges between $2 and $16 per kg (Price et al. 2020) limits the applications of the final polymer blends.

Starch is one of the most abundant natural polysaccharides and can be extracted from different sources: cereals (corn, wheat, or rice), tubers (cassava or potato), or legumes (pea). According to the report on starch market production in 2017 (https://www.beroeinc.com/category-intelligence/starch-market/), the global starch production was ~85 million metric tons (MMT), out of which 53% went into sweeteners, 36% into native starches, and 11% into modified starches, shared by regions like North America 40%, Europe 21.5%, and Asia Pacific 30%. The key production countries for types of starch were as follows: corn starch, the USA with 30%; tapioca starch, Thailand 35.7%; and wheat starch, Europe with ~20%. The leading suppliers for commercial starch were considered ADM and Cargill (USA) for corn starch (the price of ~14.36 $/hundredweight (CWT)), Siam Quality Starch Co Ltd and General Starch Ltd (Thailand) for tapioca starch (the price of ~356 $/MT), and ADM and Roquette Freres (France) for wheat starch (~410 $/MT). As for native and modified potato starch suppliers, Novidon and Avebe (The Netherlands), AGRANA (Austria), Ingredion Incorporated (USA), and Manitoba Starch Products (Canada) are some important players on the market. The difficulty of starch processing due to the inter- and intramolecular hydrogen bonds in starch macromolecules can be exceeded by adding a plasticizer to improve its processability and obtain thermoplastic starch (TPS). The plasticizers such as glycerol, water, glucose, fructose, and sucrose are usually used (Teixeira et al. 2007). Due to its colorless, transparent, odorless, and nontoxic properties, glycerol is a classical plasticizer for starch (Rhim et al. 2013). Starch sources, additives, processing parameters, and chemical treatment of starch influence its thermal, mechanical, barrier properties, moisture sorption behavior, and surface tension, which define the final applications of TPS: compost bags, coatings, packaging materials, mulch films, and disposable diapers (Zhang et al. 2014). TPS has several unfavorable properties such as high shrinkage in the mold, relatively weak mechanical properties, high moisture uptake, and high viscosity (Girones et al. 2012). An alternative to overcome the limitation of TPS and to retain its biodegradability is blending TPS with other biodegradable polymers such as poly(caprolactone) (PCL) (Averous 2000; de Campos et al. 2013), poly(vinyl alcohol) (PVA) (Fahma et al. 2017), or PLA (Jullanun and Yoksan 2020). Another important application of commercial starch is a biomedical field, due to its biocompatibility and chemical stability, particularly in wound management, drug delivery, and tissue engineering applications. This chapter summarizes the traditional and emergent technologies for the processing of some representative commercial bioplastics, highlighting the processing parameters and the main advantages/disadvantages of each technology.

4.2 Processing of Commercial Bioplastics

Although not all bioplastics are biodegradable, they are derived from renewable resources and by modification of their properties with suitable additives, improved mechanical and thermal properties, biodegradability, and/or biocompatibility, as well as easy processing at an industrial scale can be achieved. The most typical technologies available for processing of the selected representative commercial bioplastics available on market, together with their properties modifications, are outlined in Table 4.1. Therefore, injection molding, compression molding, melt blending and single/twin-screw extrusion, blown extrusion, thermoforming, 3D printing, electrospinning, as well as common processing techniques such as solution casting (solvent evaporation) and coating were applied to obtain the bioplastic specimens or other materials having improved properties compared with petrochemical polymers.

4.2.1 Injection Molding Technology

Different PLA, PBAT, PBSA, and starch systems processed by injection molding and their related properties are summarized in Table 4.1. According to data shown in Table 4.1, the technological steps necessary prior to injection molding technology are the drying of biopolymers and the compounding of blends. The use of biomass and mineral fillers such as talc and clay in formulations with the purpose to increase the crystallinity of blends and to decrease the cost of the injection molding process

Table 4.1 Some examples of commercially bioplastic compositions, their processing, and properties

Composition of bioplastics	Technology/ technological steps	Processing parameters	Properties	Reference
Thermomechanical processing (Injection molding, melt mixing/extrusion, blow molding, thermoforming)				
PLA (Ingeo 4060D and 2500HP NatureWorks)/ PBAT (Ecoflex® F Blend C1200) or PBSA (Bionolle 3001 MD) nanoclay (Cloisite 30B)	Compounding in a co-rotating twin-screw extruder (TSE), screw diameter (D) of 18 mm and L/D ratio of 40; Injection-molding	Pressure of 1500 bar;	75PLA/25PBAT blends—increase in PLA crystallization and ductility up to ~205%; more homogenous blend morphology	Nofar et al. (2019)
	Blend ratios of 74/25/1 and 48.5/48.5/3	Barrel temperature profile of 170/190/200/200/ 200 °C		
80% PLA (Ingeo Biopolymers 2500HP + 3260HP, NatureWorks)/20% PBSA (Bionolle 3020 MD)/(2–30%) talc (Luzenac HAR W 92)	1. Drying of PLA and PBSA;	1. Drying 8 h, in a vacuum stove at 45 °C, 150 mbar until the humidity level <250 ppm	Crystallinity degree increases with talc content up to 30.2% (nucleating effect of talc); Blends with talc >15%—improved performance during the brewing tests in instant coffee machines	Barletta et al. (2019)
	2. Compounding in a twin-screw extruder (24 mm screw diameter);	2. Temperature profile was 155/185/195/195/190/ 190/185/175/170/165 °C; screw speed 300 rpm;		
	3. Injection molding	3. Temperature barrel 225–240 °C; injection cycle 6 s; speed 100 cm^3/s		
Thermoplastic cassava starch (TPS) (Tong Chan Registered Ordinary Partnership, Thailand)/PLA (Ingeo™ Biopolymer 3052D, NatureWorks,	1. Cassava starch, CP, glycerol, citric acid—mixed in a ribbon mixer; TPS/CP masterbatch (50:50:35 wt% TPS:CP: glycerol and 0.5 wt% citric acid)—melt	1. Twin-screw extruder: L/D ratio of 40, barrel temperature 80–160 °C, screw speed 280 rpm, material feed rate 40–50 rpm;	22.1 wt% CP—reinforcement for TPS/PLA blend (enhanced tensile strength up to 354% and Young's modulus up to 722%); CP—nucleating agent for PLA (reduced	Jullanun and Yoksan (2020)

USA)/cassava pulp (CP)	blended in a twin-screw extruder;		Tcc), reduced melt flowability and increased shear viscosity of the TPS/PLA blend; CP (4.4, 8.8, and 13.3 wt %)—droplets in a matrix for TPS/PLA/CP composites and a bicontinuous phase structure for CP 17.7 and 22.1 wt%	
	2. TPS/CP masterbatch, TPS, and PLA pellets fed in a twin-screw extruder	2. Temperature 85–175 °C, screw speed 280 rpm, material feed rate 8 rpm (2.5 mm-length pellets);		
	3. injection molding	3. Temperature 140–245 °C, back pressure 3%, cooling time 14 s		
PLA (Ingeo™ 8052D, NatureWorks)/10 wt% bio-based polyethylene terephthalate (PET) composite foam	1. Drying of pellets	1. PLA—dried 6 h at 75 °C, PET dried 8 h at 135 °C;	Izod and Gardner impact strengths of the composite foam—more than sevenfold and sixfold higher than those of the PLA foam fabricated by regular microcellular injection molding; Expansion ratio varying from 3.8-fold to 26.2-fold; Thermal conductivity of composite foam was 26.8 mW/mK	Wang et al. (2020)
Medical grade CO_2 used as blowing agent	2. Compounding using a twin-screw extruder with a diameter of 26 mm and aspect ratio screw of 40-fold	2. Temperature (hopper to die) 60, 150, 180, 210, 210, 210, 210, 200, 200, 180, and 180 °C; feeding rate 12 kg/h; screw speed 120 rpm;		
	3. Melt spinning	3. Cooling chamber temperature 165 °C; rolling speed 850 rpm;		
	4. High-pressure microcellular injection molding (HPMIM)	4. Melt temperature 190 °C, mold temperature 45 °C, injection speed 60 cm^3/s and gas dosage 6.0 wt%		

(continued)

Table 4.1 (continued)

Composition of bioplastics	Technology/ technological steps	Processing parameters	Properties	Reference
PLA (2003D)/LAPOL 108/PEG (BioUltra 4000)/5% collagen hydrolysate (HC)/ commercial silver nanoparticles (AgNPs) 0.5%	Extrusion on single-screw extruder (Ø 12, L/D ratio = 9:1), speed 20–200 rpm; nozzle φ 4 mm, mandrel 3 mm	Speed from 20 to 60 rpm;	The presence of HC led to a marked decrease in flexibility;	Rapa et al. (2019)
		Temperature from 139 to 150 °C	Biocomposites showed a high degree of biocompatibility allowing the selection of a promising biomaterial for antimicrobial urinary drain	
PLA (2002D)/PBAT (KD-1024)/0.75 wt% Joncryl ADR-4370S;	1. Drying of pellets	1. PLA and PBAT dried under vacuum at 60 °C for 12 h;	The elongation at break was 579.9%; The notched impact strength was 29.6 kJ/m^2	Wang et al. (2019b)
PLA/PBAT ratio = 60/ 40	2. Reactive blending by melt mixing	2. Temperature 180 °C for 10 min, screw speed 60 rpm;		
	3. Hot-pressed to obtain sheets with thickness from 1 to 4 mm	3. Temperature 180 °C, pressure 10 MPa and pressing time 10 min		
PLA (6201D)/ polypropylene carbonate (PPC)/PEG-polyhedral oligomeric silsesquioxane (POSS)	Reactive melting extrusion catalyzed by Sn(Oct)2	Extrusion temperature 180 °C/190 °C/200 °C/ 205 °C/210 °C in extruding zones, respectively, the screw; the extrusion process was under vacuum	The compatibility of composites and mechanical properties were improved. The best performance was obtained in the presence of 20–40% PPC and 4% PEG-POSS in composites	Chen et al. (2020)

PLA/PBS/talc/Vitamin E/processing additives and PLA/PHB/talc/Vitamin E/processing additives nano-composite film	1. Compounding with twin-screw co-rotating extruder	1. The compounding extrusion process was set at temperatures of 150/175/185/185/190/190/185/175/165/160 °C and the screw speed was 400 rpm	The nano-composite film containing PHB showed a significant improvement of gas barrier property	Aversa et al. (2020)
	2. Cast extrusion by cast extruder with a two-roll calender			
70 wt% PLA (4043D, NatureWorks)/30 wt% PBSA (BioPBS™ FD92PM)/20 wt% thymol blend film	Blown film extrusion with a single screw extruder connected with a 34 mm diameter annular die to form a film	For feeding, compression, metering, and die zones, the temperature profile used was 100 °C, 150 °C, 155 °C, and 165 °C, respectively. Rotation rates of feeder and nip roll were fixed at 50 rpm (0.09 G) and 0.04 m/s, respectively	The films were effective against fungal growth of *Aspergillus* spp. and *Penicillium* spp., both in vitro and in packed bread; The shelf life of bread extended with 9 days compared to 3 days in the case of commercial BOPP	Suwanamornlert et al. (2020)
PLA (4044D, NatureWorks)/poly (butylene succinate-co-adipate) (PBSA) (BionolleTM 3001MD)/Joncryl® ADR 4368 (ESA)	1. Drying of pellets;	1. Drying at 85 and 50 °C, 4 h;	The improvement in mechanical, optical, barrier and antislip properties of the films was explained by the formation of long-chain branching (LCB) structure due to the incorporation of chain extender (ESA)	Palai et al. (2020)
	2. Premixing in dry blender;	2. Rate 80 rpm and 50 °C temperature for 10 min;		
	3. Compatibilization of premixed blends using co-rotating twin-screw extruder (L/D ratio 25:1)	3. 80–100 rpm; temperatures 155/160/165/170/170/175/175 °C (feed zone to die zone);		
	4. Extrusion blown film in an extruder with diameter of 32 mm, L/D ratio of 28:1 and a gear reduction ratio of 12.5:1	4. Temperature of the barrel 160/160/165 ± 5 °C; lay flat width 300 mm; bubble diameter 191 mm; die gap 0.8 mm; blow-up ratio 2.54; drawdown ratio 6.3; forming ratio 2.48		

(continued)

Table 4.1 (continued)

Composition of bioplastics	Technology/ technological steps	Processing parameters	Properties	Reference
PLA (4032D, NatureWorks)/PBAT (Ecoflex FBX 7011)/ Joncryl ADR®-4368	1. Drying of pellets	1. Vacuum at 80 °C for 12 h;	The addition of Joncryl to PLA-PBAT blend led:	Al-Itry et al. (2015)
	2. Compounding	2. Temperatures 140 °C (feeding zone), 190 °C, 180 °C (melting zones) and 180 °C (die); screw rotation speed 40 rpm for 3 min;	• to decrease the interfacial tension,	
	3. Blowing extrusion	3. Annular 25 mm diameter blow die coupled to a melt pump and twin extruder; the temperature of the die was 180 °C	• to improve shear and elongational rheological properties,	
			• to enlargement of the blowing processing window,	
			• to reduce the instability defects,	
			• to improve the thermomechanical properties of blown films	
PLA (2002D, NatureWorks) for rigid film	1. Single-screw extruder (screw diameter 45 mm, length/diameter ratio of 37); extrusion die width 600 mm—Flat film (500 mm wide, 0.3 mm thick)	1. Temperatures 180, 185, 190, 195 and 200 °C; gear pump rotational speed 100 rpm;	The biodegradation process is less dependent on the thermoforming process of PLA and more dependent on the composting/degradation conditions that are applied	Musioł et al. (2016)
	2. Thermoforming. Tray with	2. Temperatures heating 240 °C for all central zones and 280 °C for external zones; temperature of		

	30 × 120 × 155 mm (depth, width, length)	cooling 50 °C; cooling time 15 s; holding time at lower pressure 8 s and thermoforming cycle 45 s		
PLA (PLLA L175, Total Corbion)/PBS (Bio PBS FZ91)/ mineral fillers	1. Reactive extrusion (co-rotating twin-screw extruder), screw diameter of 27 mm;	1. Temperature profile was set at 160/170/175/180/185/180/180/175/175/170 °C	20 wt% $CaCO_3$ in the formulation ensured greater crystallinity, reduced water absorption in the material, the most uniform stretching ratios in the thermoforming process and low-cost	Barletta and Puopolo (2020)
	2. Cast extrusion (flat head extrusion laboratory plant)	2. Temperature profile was set at 170/200/205/180/170 °C and screw speed was 180 rpm		
	3. Thermoforming food containers	3. Heating time in the range of 13–20 s and vacuum time was 20 s and 25 s, respectively		
Corn starch (Sigma Aldrich)/PBAT (Ecoflex®, BASF)/ biomass (30–70 μm) (60/20/20 wt%)/lignin	1. Drying in a vacuum oven	1. Starch and the filler materials were dried 6 h at 80 °C, Ecoflex® was dried 4 h at 60 °C	Blend compatibility—relatively good; biomass fillers increased water absorption capacity; Lignoboost lignin improved the tensile and impact strengths, decreased water uptake capacity, and offered good antimicrobial properties against *S. aureus* and especially *E. coli.*; DSC: biomass wastes inhibit the crystallization of the starch/Ecoflex® material	Spiridon et al. (2020)
Biomass: celery fibers, poplar seed hair fibers, pomace, *Asclepias syriaca* fibers	2. Melt compounding in fully automated Brabender station	2. 150 °C for 10 min, 60 rpm;		
	3. Compression molding in Carver press	3. 140 °C (pre-pressing step of 3 min at 50 atm and a pressing step of 2 min at 150 atm)		

(continued)

Table 4.1 (continued)

Composition of bioplastics	Technology/ technological steps	Processing parameters	Properties	Reference
Native cassava starch (CS) (Guangxi Nanning Jinguang Starch Co. Ltd., China)/ glycerol and nano-silica (nano-SiO_2, 20 nm of primary particle size)	1. Nano-SiO_2 dispersed in glycerol by ultrasonication;	1. 90 min under 50 °C, power of 200 W	Increase of glycerol (40 phs [parts of glycerol per 100 parts of dried starch])—improved elongation at break (up to 108%), less stiffness; 25 phs glycerol (poor plasticization)—difficult extrusion of TPS film—cannot be prepared by film blowing; Addition of 1 phs nano-SiO_2: tensile strength increased ~95%; increase of glycerol and nano-SiO_2—decreased CS crystallinity, increased thermal stability; SEM: trapezoidal layered structures (complete plasticization under high glycerol) (45 and 50 phs)	Liu et al. (2020)
	2. Glycerol containing nano-SiO_2 mixed with the dried CS in a high-speed mixer;	2. Set at room temperature 24 h		
	3. Twin-screw extruder (TPS/nano-SiO_2 masterbatch);	3. Screw L/D ratio of 42:1, screw diameter of 35.5 mm, the screw speed about 480 rpm in the temperature range of 140–165 °C		
	4. TPS/nano-SiO_2 film (a single-screw extruding film-blowing machine)	4. Screw L/D ratio of 34:1, screw diameter of 28 mm in the temperature range of 120–160 °C		
Thermoplastic cassava starch (Dragon Fish brand, Tong Chan, Thailand)/chitosan	1. Solution mixing of cassava starch, chitosan (CTS), and glycerol in acidified water (lactic	1. Cassava starch was mixed with glycerol (70/30) and 200 mL distilled water in a water bath at 80 °C for 30 min; Sodium benzoate (BEN) or	Tensile strength and elongation at break of TPSC/ENR increased with the additive	Kodsangma et al. (2020)

(TPSC)/epoxidized natural rubber (ENR)	acid 1 wt%) at 80 °C followed by melt mixing at 130 °C;	chlorhexidine gluconate (Cl) were incorporated during solution mixing (1, 5, 10 wt%)	content; addition of Cl—elastic recovery improved; FTIR: reaction between CTS reactive functional groups and the additives with the epoxy groups of ENR led to improved mechanical properties, elasticity, morphology, antimicrobial properties of TPSC/ENR/additives blends	
	2. TPSC, TPSC/BEN, and TPSC/Cl combined with ENR by melt mixing (70/30 wt/wt)	2. Two roll mill at 130 °C for 10 min. (Sheets obtained by hot compression at 130 °C for 5 min)		
3D printing method				
PLA (New Verbatim)	3D printing	Nozzle temperature 240 °C, Build-plate temperature 60 °C, Print speed 30 mm/s, Shell thickness 0.4 mm, Layer height 0.1 mm, Infill density 100%	Young's modulus 3479 MPa, 0.2% proof stress of 41.7 MPa, ultimate tensile strength (σ_{UTS}) 42.9 MPa	Ezeh and Susmel (2020)
PLA/carbon fiber	3D printing based on FDM machine	Infill density 80%; Print speed 80 mm/s; Layer height 100 m	Optimum tensile strength of 21.961 MPa	Ajay Kumar et al. (2020)
PLA/15% carbon filaments of 1.75 mm diameter obtained by extrusion	FDM in 3D printing	0.2 mm layer thickness, air gap 0.4 mm, 90° orientations, 220 °C temperature with TiC nozzle	Tensile strength of 80 MPa after chemical treatment of tensile specimens with acetone of 100% concentration for 120 s, Tensile strength of 74 MPa (heat treatment process 120 s at 120 °C)	Guduru and Srinivasu (2020)

(continued)

Table 4.1 (continued)

Composition of bioplastics	Technology/ technological steps	Processing parameters	Properties	Reference
SMARTFIL® PLA natural, SMARTFIL® PLA 3D850 natural and HDPlas® PLA (PLA-graphene) filaments (1.75 mm)	3D printing based on FFF	Layer thickness of 0.12 mm; Feed rate 50 mm/s; Flow rate 4.8 mm^3/s; Top and Bottom thickness 0.6 mm; Nozzle temperature 210 °C; Nozzle size 0.4 mm	PLA-graphene composites—improved tensile and flexural stress, the highest interlaminar shear strength; reduced impact strength, without affecting the dimensional accuracy of specimens	Caminero et al. (2019)
PLA (Ingeo 3260HP, NatureWorks LLC)/ PHA copolymer Mirel grade (Metabolix USA) (80/20)	3D printing based on Fused layer modeling (FLM)	Blends compounded in a twin-screw extruder Brabender TSE 20. Specimens obtained with a printing nozzle (diameter 0.6 mm), the temperature of 205 °C, printing bed temperature of 80 °C, print speed 50 mm/s, layer height 0.3 mm	Notched impact strength values increased from 6.5 J/m^2 in the case of pure PLA to 12.7 kJ/m^2 for PLA/PHA blend	Ecker et al. (2019)
PLA/ABS (80/20; 50/50; 20/80%)	3D printing	Infill density 50%; Feed rate 65 mm^3/s; Nozzle diameter 0.4 mm	High resistance to strength	Dhinesh et al. (2020)
50 wt% corn starch (Max's, Holland; ~10 μm), 30 wt% dextran (Sigma, USA; >100 μm) and 20 wt% gelatin (>100 μm) (three-powder blend)	3D printing (Zcorp Z402)—five scaffolds designed using a CAD software (Unigraphics v. 15.0.3) and uploaded to the 3DP software where the model was sliced by a slicing algorithm. 2D sliced layers were built by the	Scaffolds dried 1 h at 100 °C after printing. One batch was infiltrated with different amounts of 2% copolymer solution (75% poly(L-lactide) acid and 25% PCL (Gunze, Japan) in dichloromethane), then evaporate and dried at 27 °C; the second batch of scaffolds—infiltrated by the same method, but after drying, scaffolds were soaked in deionized water for 10 min and dried at 100 °C	Infiltrated scaffolds–decreased microporosity, improved mechanical properties; more resistant to water absorption with increasing copolymer volume; DSC results—chemical bonding and	Lam et al. (2002)

	printer layer by layer till the final object was physically formed		uniform blending of materials	
Native cassava starch (Amilogill 1500, Cargill Agrícola—Brazil)	Starch modification by ozone technology (starch oxidation); selected gels (10.7 g starch/100 g) were processed in a 3D printer (Stampante)	Printer syringes (60 mL) containing starch gels, closed with parafilm were stored for 7 days in a refrigerator (5 ± 2 °C), then immediately 3D printed (0.8 mm diameter nozzle, nozzle height of 18 mm, speed of 20 mm/s and an extrusion rate of 30 mm/s at 20 °C). A star model was created using the Repetier Host V2.0.1 and Slic3r software	Ozonation time increased—starch with lower pH and molecular size (depolymerization), higher carbonyl and carboxyl contents, gels with different behaviors (stronger and weaker than the native ones, depending on processing time); Gels produced by native and starches ozonated for 30 min—good printability (at lowest gelatinization temperature, 65 °C); star produced with the ozonated starch—better resolution (well-defined angles)	Maniglia et al. (2019)
Gelatinized starch (Great Value)/ Hydroxyapatite (HA) powder; Polycaprolactone (PCL) pellets (MW 14,000)	Ceramic slurry-based solid freeform fabricator (SFF)	SFF print head module (244 mm height × 88 mm length × 94 mm width), mounted on a gantry of a conventional 3D printer; extruder motor (motor with holding torque 45 N cm); piston cylinder (PC) nozzle 0.5–0.9 mm, layer height 1.5 mm, shell thickness 0.5 mm; Infill (40–70%) and	Gelatinized starch—reinforcement phase for HA composite scaffolds; starch loading improved mechanical strength from 4.07 ± 0.66 MPa to	Koski et al. (2018)

(continued)

Table 4.1 (continued)

Composition of bioplastics	Technology/ technological steps	Processing parameters	Properties	Reference
were used for two scaffolds		printing speeds 40–70 mm/s; scaffold 20 mm height, 11 mm diameter	10.35 ± 1.10 MPa (~mechanical strength of cancellous bone); SEM and MTT cell viability assay—enhanced human osteoblast cell proliferation in vitro in the presence of starch and PCL	
Commercial blend of corn starch/poly-(ε-caprolactone) (SPCL) (30:70 wt%)	Supercritical fluid technology (supercritical assisted phase inversion) to produce 3D scaffold, highly porous and interconnected	15 wt% SPCL solution in chloroform; 2 mL loaded in a stainless steel cap (2 cm diameter), inside the high pressure vessel heated in by an electric thin band heater; CO_2 is pumped into the vessel using high pressure piston pump until operational pressure; system closed for 45 min to allow the phase separation, flushed for 45 min, with a stream of CO_2 at very low flow rate (5 g/min) for complete drying of the scaffolds	Enzymatic hydrolysis by α-amylase and lipase (synergistic effect of both enhanced degradation)—weight loss of the samples incubated for 1, 3, 7, 14 and 21 days; micro-computed tomography (m-CT) and surface chemistry analysis results—SPLC scaffolds undergo bulk degradation (hydrolysis of chemical bonds in the polymer chain at the centre of the matrix, resulting in a highly porous material)	Duarte et al. (2010)

Electrospinning technique				
PMS (poly mannitol sebacate)/PLA nanofibers with different polymeric weight ratios of 3:2, 1:1, and 2:3	Electrospinning	The flow rate injection 1 mL/h; the distance between needle and collector 20 cm; the voltage 21 kV and the rate of drum collector ~400 rpm	By applying thermal crosslinking, the average ultimate tensile stress (σ_{UTS}) increased from 2.51 to 3.88 MPa to 6.08–11.07 MPa and Young modulus (E) increased from 47.88 to 57.10 MPa to 101.37–142.31 MPa	Rahmani et al. (2020)
PLA Ingeo 4043D and zein-CP2010/rosemary essential oil (10 and 20 wt%)	Electrospinning and extrusion	Flow rate 1 mL/h, voltage 27 kV, and target distance 15 cm; The optimum extrusion parameters for PLA: 50 rpm and 180 °C, while for zein the screw rotation speed was 40 rpm, and the temperature was 110 °C	Maxim encapsulation efficiencies were 94.7% and 87.1% by electrospinning for PLA matrix and zein and 72.6% and 69.8% by extrusion for the same matrices	Stramarkou et al. (2020)
High amylose maize starch (HAMS), (Gelose 80, Ingredion, US) and cationic starch (CS), (Chargemaster R33F, Grain Processing Corporation, US)/ nanocellulose (NC) (150–200 nm length, 5–10 nm diameter)	Composite fiber mats formed by electrospinning	Flow rate of 6–12 mL/h; Spinning distance of 5–8 cm; Voltage of 6–10 kV	Starch electrospinnability—deteriorated by an increase in branched amylopectin fraction; due to high shearing during electrospinning effective interactions (ionic bonding CS-NC, intra- and intermolecular H bonding, and	Wang et al. (2019a)

(continued)

Table 4.1 (continued)

Composition of bioplastics	Technology/ technological steps	Processing parameters	Properties	Reference
			macromolecular entanglements) provide structural support and facilitate fiber formation; CS-NC increase the tensile strength of the starch fibers	
Starch (Hycel de Mexico)/PLA, treated with arginine-glycine-aspartic acid peptides (RGD) scaffolds	Electrospinning	Solution of PLA in chloroform (8% w/w) with starch (0, 2.5, 5.0, 10.0%) mixed 24 h, 35 °C prior to electrospinning, then supplied with constant flow (*Q*: 2 mL/h, syringe pump, TL-F6) to a metallic capillary (OD 0.71 mm, ID 0.41 mm, length 40 mm) connected to a high voltage source (0–50 kV) into the electrospinning device TL-01; voltage of 6 kV, 15 cm distance tip of the capillary—metal collector (rotor speed 100 rpm)	Functionalization with RGD—enhanced wettability that favors the adhesion, propagation, and proliferation of osteoblasts cells; Increased starch amount—more irregular surfaces	Gutiérrez-Sánchez et al. (2019)
		Casting method		
PBAT (Ecoflex, BASF)/5, 10, and 15 wt % orange essential oil (OO)	Film casting	1.4 g of the PBAT dissolved in 50 mL chloroform (solvent); PBAT initially dissolved for 30 min, then OO added in dissolved PBAT and mixed for 30 min (total constant stirring: 45 min); poured solutions were allowed to evaporate for 48 h	SEM: pores on the film surface; improved homogeneity with increasing OO amount; OO reduced *E. coli* growth rate	de Andrade et al. (2020)

Thermoplastic cassava starch (Alini brand)/PVA composite films with cellulose nanofibers (NF) as reinforcement agent and glycerol as plasticizer	Film casting	Gelatinization of granular starch—cassava starch mixed with PVA (4:1) (stirring, up to 90 °C for 10 min) and dissolved in water. Glycerol (0 and 25 wt%) and cellulose nanofibers (0, 1, 3, and 5 wt %) were added to the suspension, stirred for 15 min. The resulted material was molded in Teflon mold and dried in the oven at 45 °C for 48 h	Addition of NF until 3 wt% increased tensile strength and films crystallinity, but decreased elongation at break and water vapor transmission rate (WVTR). Glycerol increased films elongation at break and WVTR but lowers tensile strength	Fahma et al. (2017)

was also evidenced in Table 4.1. For example, nanocomposites based on Ingeo 4060D and 2500HP, poly((butylene adipate)-*co*-terephthalate) (PBAT) (Ecoflex® F Blend C1200), poly((butylene succinate)-*co*-adipate) (PBSA) (BIONOLLE 3001 MD), and nanoclay (Cloisite 30B) were prepared through an injection-molding (IM) process with the attempt to improve the ductility and processability of PLA (Nofar et al. 2019). The authors showed that the Ingeo 4060D/PBAT blends exhibited an increase in ductility of ~205% from ~5%, while the incorporation of 1 wt% nanoclay into the PLA/PBAT blend led to increasing the ductility to only 8%. In the case of the PLA/PBSA blend, the ductility was lower due to heterogeneous morphologies. In another paper (Barletta et al. 2019), Ingeo Biopolymer 3260HP and Ingeo Biopolymer 2500HP (NatureWorks LLC), two commercially PLAs, designed for extrusion and injection molding, respectively, in equal amounts, were compounded with poly(butylene) succinate co-adipate (PBSA) (Bionolle 3020 MD) and talc and further used for manufacturing of coffee capsules by injection molding. It was reported the significant role of talc in the dimensional stability of coffee capsules by limiting the capability of PLA blends to recrystallize, validated by brewing tests in instant coffee machines. Chiu et al. (2013) performed the compounding of PLA 2100D grade from Nature Works in the presence of PBAT (Ecoflex 7011) from BASF for the obtaining of specimens from injection-molding technology. Immiscible blends with the best tensile and impact properties were reported for 30/70 PLA/PBAT, due to sea-island morphologies. The increase in the crystallinity degree and heat deflection temperature (HDT) was achieved by annealing of PLA/PBAT samples at 100 °C for 8 and 24 h. The foaming characteristics of PLA 4032D compared to those of acrylonitrile butadiene styrene (ABS) were investigated by Seo et al. (2012), using an injection molding process. The combining effects of chemical blowing agents (1%, 2%, 3%, and 10%) and nitrogen for the microcellular foaming process (MCP) permit to achieve a higher foaming ratio. In this way, the mechanical properties do not deteriorate. This process can be applied to manufacture lightweight materials, which show good mechanical properties. High-performance multifunctional porous materials with potential applications in auto, aerospace, transportation, and packaging have been obtained by high-pressure microcellular injection molding (HPMIM) of composite based on commercially PLA (Ingeo™ 8052D) and bio-based PET (BioPET 001) (Wang et al. 2020). HPMIM is an innovative technology that permits the extruded PLA/PET composite to stretch and spun into PLA/PET fibers with the help of a self-designed melt spinning apparatus.

Injection molding can be also used to form capsules or other shaped products containing starch made from corn wheat, potatoes, rice, and tapioca with molecular mass in the range of 10,000–20,000,000 Da (Wittwer and Tomka 1984). Different compositions of commercial starch with water (5–30 wt%) or extenders (sunflower proteins, peanut proteins, rapeseed proteins; soybean proteins, cotton seed proteins, blood proteins, egg proteins, water-soluble derivates of cellulose, polysaccharides, polyvinylpyrrolidone; bentonite, polyvinyl acetate-phthalate; vinyl acetate, gelatin, silicates, etc.) or starch mixed with one or more plasticizers (0.5–40 wt% based on starch weight) such as polyethylene glycol and low-molecular-weight organic plasticizers (for example, glycerol, sorbitol, dioctyl sodium sulphosuccinate, triethyl citrate, tributyl citrate, 1,2 propyleneglycol, mono-, di-, and tri-acetates of glycerol) can be used. For injection molding, plasticizing the starch composition should be performed at a temperature in the range from 80 to 240 °C, then injecting the plasticized composition into a mold; the injection pressure is preferably in the range from 600×10^5 to 3000×10^5 N/m^2. Blending of PLA with thermoplastic cassava starch (TPS) imparts reasonable cost to the final PLA/TPS blend and reduces the brittleness of PLA (Yokesahachart and Yoksan 2011). Continuous melt processes, i.e., twin-screw extrusion and injection molding, have been used to prepare biodegradable TPS/PLA (60/40 wt%) blends containing 2.7, 5.5, 8.2, and 10.9 wt%. coir fibers (CF) of 3–7 mm length (Chotiprayon et al. 2020). Pellets of TPS were obtained by compounding the premix of cassava starch (Dragon Fish brand, Tong Chan Registered Ordinary Partnership, Thailand) and glycerol in a twin-screw extruder (L/D ratio of 40, barrel temperature in the range of 80–150 °C, screw speed of 280 rpm), the final TPS/PLA/CF composites being realized by means of a twin-screw extruder (L/D ratio of 40, barrel temperature in the range of 95–165 °C, screw speed of 200 rpm). Dumbbell- and bar-shaped composites specimens were processed using an injection molding machine (temperature range of 140–170 °C, injection speed of 5–28%, a maximum injection pressure of 42–45 bar, and a cooling time of 30 s). Temperature-dependent FTIR indicated hydrogen bond formation between the cassava starch and PLA (Ingeo™ biopolymer 3052D) and/or CF. Results showed that CF reinforced the TPS/PLA blend, improved the dispersion of TPS and PLA phases, and played the role of nucleating agent for PLA in the blend, although CF reduced the melt flowability and decreased the tensile strength, extensibility, and impact strength of the TPS/PLA blend. TPS/PLA/CF composites could be used for obtaining renewable, biodegradable, and eco-friendly injection-molded products including packaging (e.g., tray, box, cup), toys, and stationery.

Microalgae biomass with a high protein content (~48%) (López Rocha et al. 2020), fish proteins (Araújo et al. 2018; Uranga et al. 2019), and wheat gluten (Jiménez-Rosado et al. 2019) were found as a great potential solution for manufacturing of commercial bioplastics due to some advantages in term of scale production and cost. Microalgae biomass was mixed with glycerol at different ratios (50%, 55%, and 60%) and processed by injection molding at a temperature of 140 °C, injection time of 5 min, and injection pressure of 500 bar (López Rocha et al. 2020). The specimens showed high thermal stability and lower water absorption.

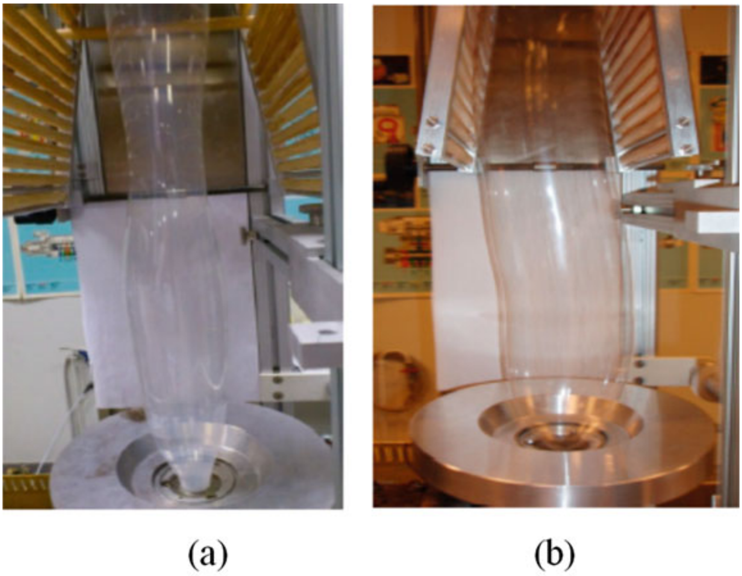
4.2.2 Extrusion Technology

Rapa et al. (2019) reported the processing of bionanocomposites based on Ingeo™ Biopolymer 2003D, polyethylene glycol (PEG 4000) and Lapol 108 (as plasticizer and compatibilizer), collagen hydrolysate, and silver nanoparticles (AgNPs) by extrusion technology at laboratory scale for manufacturing of potential antimicrobial urinary drains. The introduction of collagen hydrolysate into PLA-based

Fig. 4.1 Flexible tube obtained by extrusion of PLA bionanocomposite formulation. (Reprinted with permission from Rapa et al. 2019)

formulations led to obtaining tubes with nonuniform and stiffer surfaces, while the smooth and glossy tube surfaces with an inner diameter of 3 ± 0.1 mm and outer diameter of 4 ± 0.1 mm were achieved in the case of formulation without hydrolyzed collagen (Fig. 4.1). The manufacturers of PLA films at an industrial scale should envisage different strategies to improve the melt strength and overlap the brittle properties of PLA. Incorporation of chain extenders (under the name Joncryl) and the blending of PLA with other polymers are two routes used for overlapping the main drawbacks of PLA. For example, films with a thickness of 0.05 mm and adequate propertied for packaging applications were obtained at laboratory scale using blown film extrusion process by blending of PLA 4044D grade with poly (butylene succinate-co-adipate) (PBSA) and epoxy functionalized styrene acrylate (ESA) as chain extender (Palai et al. 2020). The improvement in the gas barrier properties of PLA has been demonstrated by Aversa et al. (2020) by three strategies: (1) incorporation of the inorganic filler (talc); (2) adding of immiscible biodegradable polymers, such as polybutylene succinate (PBS) and poly-3-hydroxybutyrate (PHB); and (3) introduction of an antioxidant agent, tocopherol (vitamin E), into the polymeric matrix. In this context, films 180 mm thick and 100 mm wide were manufactured by cast extrusion of PLA/PBS and PLA/PHB loaded with tocopherol, talc, and processing additives (Aversa et al. 2020). Low oxygen permeation concomitant with maintaining the thermomechanical properties of sheets can be explained by the antioxidant agent's action to delay any degradation effect of the polymeric melts during the compounding and cast extrusion. In a recent study performed by Luchian-Lupu et al. (2020), it was found that the PLA 2003D grade/styrene-isoprene-styrene triblock polymers (SIS) compound treated by gamma-irradiation shows the improvement in material stability, thereby extending its application range on the packaging materials.

The introduction of palm leaves as natural filler up to 2% by weight into PLA PLSF 101 grade (Natureplast) was investigated by extrusion film blowing (Kharrat et al. 2020). The increase in the tensile strength and elongation at break of composites was assigned to the filler effect, which contributed to the orientation of amorphous polymer chains along the machine direction (MD). In another paper, a



(a) (b)

Fig. 4.2 (**a**) PLA modified with 0.7 wt% of Joncryl (**b**) films processed by blown film extrusion process. (Reprinted from Al-Itry et al. 2015, open access)

compound based of PLA (4032D grade) and poly(butylene adipate-*co*-terephthalate) (PBAT) (Ecoflex FBX 7011) in the presence of Joncryl ADR®-4368 was processed using a co-rotating twin-screw extruder having a length: diameter ratio of 25:1 with the purpose to be used in blown film extrusion for packaging application (Table 4.1) (Al-Itry et al. 2015). A large and stable bubble (Fig. 4.2b) has been achieved by the modification of PLA 4032D with 0.7 wt% commercial styrene-acrylic oligomers (Joncryl ADR®-4368) compared to that of pure PLA (Fig. 4.2a).

An innovative approach to ensure compatibility between the PLA matrix and nanoparticles was obtained using liquid-assisted extrusion (Herrera et al. 2017). From this perspective, PLA Ingeo 4043D grade together with a suspension of chitin nanocrystals in water, triethyl citrate (TEC) as the plasticizer and ethanol as solvent were feeding into a co-rotating twin-screw extruder. This technique permits a good dispersion of nanoparticles, due to the covering effect of the plasticizer. In another paper, Jiménez-Rosado et al. (2019) prepared wheat gluten plasticized with glyoxal or xanthan gum and water by extrusion technology at the following processing conditions: temperature of feed of 80 °C, temperatures for first mixing zone 110 °C and 120 °C, temperature for second mixing zone 120 °C, temperature for the nozzle of 100 °C, and spindle speed of 30 rpm. These blends showed greater compatibility, tensile strength, and water uptake capacity enhanced compared to the compression molding process (Jiménez-Rosado et al. 2019).

4.2.3 Thermoforming Technology

Rigid prototype packages from PLA 2002D were produced by thermoforming of PLA films previously obtained by extrusion (Musioł et al. 2016). In a comparative study between thermoforming of polyethylene terephthalate (PET) and Polysole PLA (from Solegear Bioplastics) performed by Labonté and Dubois (2015), it was found that the impact modified-PLA can successfully replace PET. For the thermoformed containers obtained using PLA, the overall molding cycle was 3.31 s, very close to that recorded by PET (3.13 s). In addition, the pressure necessary to cut the PLA shape was ~40% less than in the case of PET. For improving the thermoformability of PLA, the strategy is to add processing additives available on the market, which allow the reduction in the polymer melt's instability, its tendency to thermo-hydrolytic degradation, and the defectiveness during thermoforming (Barletta and Puopolo 2020). Three kinds of commercial mineral fillers, namely, micro-lamellar talc, titanium dioxide, and calcium carbonate ($CaCO_3$), previously dried in a dehumidifier at 50 °C for 6 h were introduced into commercial two PLA grades for manufacturing of food containers (dimensions ~150 × 150 × 50 mm^3) (Barletta and Puopolo 2020). The higher thermal diffusivity ensured by the reinforcing agent in the content of 20 wt% is assumed to give a uniform stretching ratio during thermoforming of the food containers.

4.2.4 3D Printing Technology

3D printing called "additive manufacturing" is a new technology for obtaining commercial products such as jigs, fixtures, high-precision gauges for quality control, custom car parts, and biomedical devices (Ezeh and Susmel 2020; Oladapo et al. 2020). Filament fused fabrication (FFF) also known as fused deposition modeling (FDM) is a common technique of extrusion-based 3D printed finish goods using the extrusion of filament, layer by layer (Lay et al. 2019). By 3D printing, complex shapes of prototypes, without occurring of typical waste can be manufactured. The choice of infill density, print speed, and layer height are the main parameters proving the success of manufacturing by 3D printing. The main obstacles in 3D printing are the lack of mechanical properties of filaments and the degree in crystallinity of 3D objects (Dong et al. 2019). Lay et al. (2019) showed that these drawbacks are caused by the presence of voids that appeared at the incomplete diffusion between layers. For this purpose, a good strategy for preparing feedstock filaments for 3D printing is represented by the blending of PLA with ABS (Dhinesh et al. 2020), polyvinyl chloride (PVC), wood power, and Fe_3O_4 (Kumar et al. 2019), cellulose fibers (Dong et al. 2019).

Literature data reported the use of 3D printing for producing dumbbell-shaped tensile test specimens (Caminero et al. 2019; Dong et al. 2019; Ecker et al. 2019; Guduru and Srinivasu 2020). By processing PLA (Ingeo 3260HP from NatureWorks LLC) with 20 wt% PHA copolymer (Mirel grade from Metabolix USA) by 3D-fused layer modeling (FLM) printing technology, specimens with higher impact strength compared to injection molded parts were obtained (Ecker et al. 2019). Three commercial PHAs, namely poly(hydroxybutyrate-co-hydroxyhexanoate) (PHBH X131A) purchased from Kanaeka Corporation, KITA-KU Osaka, Japan, poly (hydroxybutyrate) (PHB Hydal) provided by Nafigate Corporation, Prague, Czechia, and poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV, Mirel P1004) purchased from Telles Inc., USA. have been evaluated for their suitability for 3D-FDM (Kovalcik et al. 2020). The filaments with a thickness in the range of 1.6–1.75 mm were prepared by extrusion at 150 °C and 20 rpm, then the scaffolds were printed at a nozzle temperature of 205 °C and a printing bed temperature set to 60 °C. Compared to PLA 2003D, the PHBV scaffolds permit cell proliferation, while the PHBH X131A scaffolds show the thermal stability and rheological properties adequate for the processing by FDM. Ceramic composite scaffolds based on naturally sourced gelatinized starch with HA have been created using an additive manufacturing method, ceramic slurry-based solid freeform fabricator (SFF) (Table 4.1 and Fig. 4.3). Starch incorporation improved the mechanical strength of the scaffolds and increased the proliferation of osteoblast cells in vitro (Koski et al. 2018).

4.2.5 Electrospinning Process

Electrospinning is a hydrodynamic process for manufacturing polymeric nanofibers with unique properties via spinning of polymeric solution from a syringe under an established voltage. PLA is the most promising polyester used in combination with other biodegradable biopolymers for preparing nanofibers for tissue engineering and regenerative medicine applications. For example, Rahmani et al. (2020) prepared PLA and poly(mannitol sebacate) (PMS)/PLA nanofibers by spinning homogeneous solutions containing polymer in concentrations of 8% and 10% w/v, respectively, dissolved in hexafluoroisopropanol (HFP) solvent. The PMS/PLA nanofibers showed non-cytotoxicity by evaluation in vitro cell test and the proper degradation for use in tissue engineering without deteriorating the mechanical properties (Table 4.1). 3D scaffolds can be designed ad-hoc by electrospinning process, the resulted microstructures with high surface areas and interconnected channels (>90%) favor cell growth, transport of nutrients and metabolic waste (Gutiérrez-Sánchez et al. 2019; Salgado et al. 2004). Potato starch (Signet Chemical Corporation Pvt. Ltd., India) based nanofibrous (diameter in the range of 110–300 nm) scaffolds for wound healing applications have been obtained by electrospinning by Waghmare et al. (2018). The electrospinning parameters were: flow rate varied between 0.3 and 0.5 mL/h, the voltage varied between 15 and 37 kV, distance from the needle to the rotating collector varied between 13 and 23 cm. The incorporation of polyvinyl alcohol (PVOH) as plasticizer and glutaraldehyde as a crosslinking agent, impart durability for skin tissue engineering, the mechanical strength of 0.5–0.8 MPa being registered for starch-based scaffolds, which were found also to be nontoxic and able to promote fibroblast (L929 mouse) cellular proliferation. Stramarkou et al. (2020) performed a comparative study for the loading encapsulation of 10% and 20% *Rosemarinus officinalis* (rosemary) essential

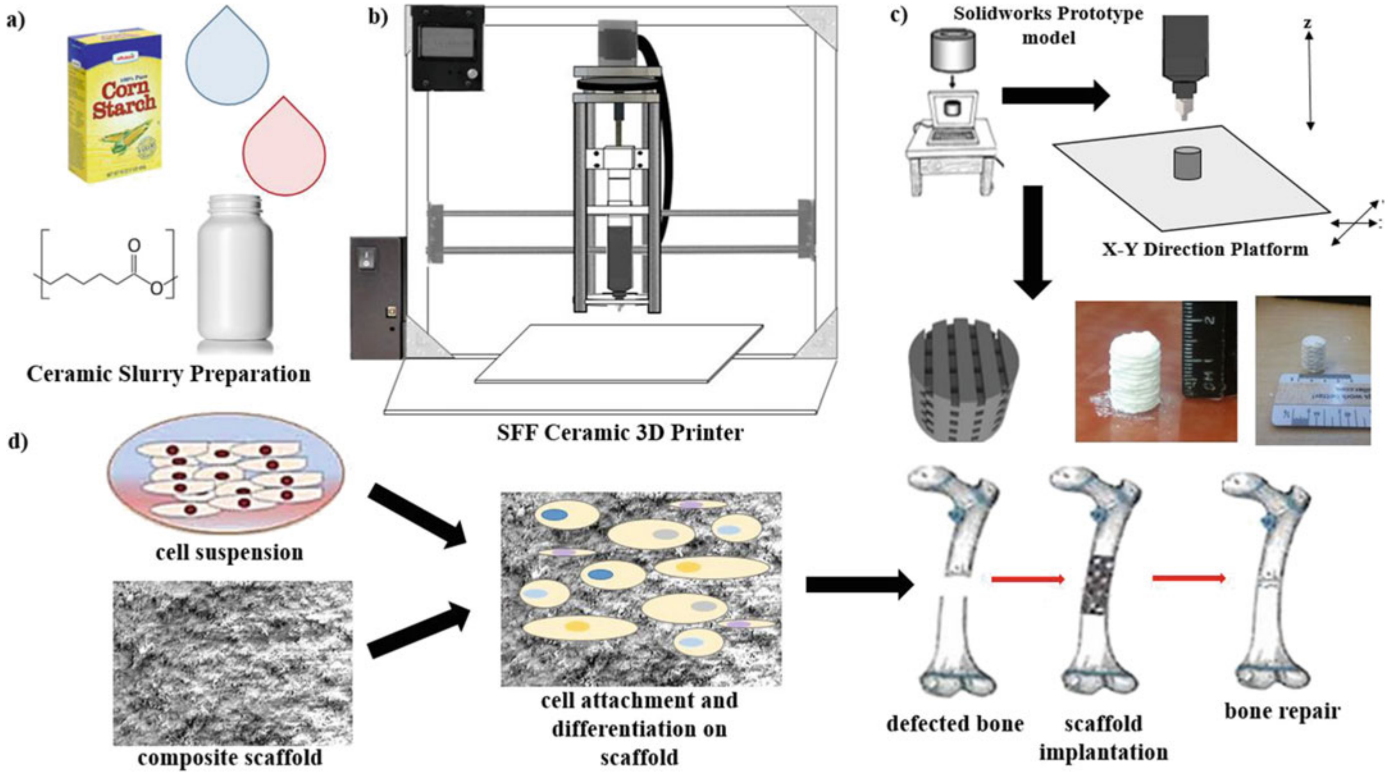


Fig. 4.3 (**a**) Ceramic slurry preparation of Starch/HA used for bone scaffolds fabrication without post-processing; (**b**) Scheme of solid freeform fabricator; (**c**) Design of scaffold model utilizing solid works file and CURA Program; (**d**) In vitro proliferation of osteoblast cells on the designed starch/HA scaffolds, possessing sufficient mechanical integrity and enhanced bioactivity to be utilized in bone repair. (Reprinted with permission from Koski et al. 2018)

oil into PLA and zein by lab-scale electrospinning and twin-screw extrusion equipments and the release rate of the bioactive agent from the obtained fibers and pellets. The results demonstrated that the PLA matrix is a more promising material for the controlled release of rosemary during 30 days as a natural pesticide and electrospinning is an appropriate process for this. Another example of flexible electrospun mats with a thickness in the range of 25–50 μm, with potential for agriculture applications, was offered by Arrieta et al. (2020), which use the PLA 3051 grade (supplied by NatureWorks®), PHB P226 grade (supplied by Biomer, Germany) and oligomeric lactic acid (OLA) (synthesized by Condensia Química S. A., Spain) as a plasticizer. According to the authors, the coaxial electrospinning parameters were set at a flow rate of 1.0 mL/h, an electrical field of ±11 kV and the distance from needle to a grounded aluminum foil collector of 14 cm (Arrieta et al. 2020). Melt electrospinning is a most promising technique for obtaining of fibers without solvent using an umbrella-like spinneret that permits a large-scale production of up to 300–600 g/h (Mingjun et al. 2019). By melt electrospinning of 6 wt% PLA under the airflow velocity of 25 m/s, the fibers with an average diameter ~256 nm were achieved.

4.2.6 Casting Method

Casting represents a laboratory-scale methodology used to prepare films of small dimensions by solution/suspension spreading that implies economical equipment and limited space (Madhumitha et al. 2018). The issues of the simple casting method refer to the long drying time (depending on the film composition and thickness), the difficulty of scaling-up to the industrial level, and the high processing cost due to low production (Yepes et al. 2019). Takkalkar et al. (2019) performed a comparative study for PLA 4032D (NatureWorks) films processed by melt processing and solvent casting methods. The differences between these two methods consist in the appearance of PLA film surface: rough surface in the case of solvent cast PLA with crystalline regions, and smooth surface, and a broad amorphous XRD peak in the case of the melt processing. The casting method is preferred to extrusion process when the dispersing of metallic nanoparticles into PLA matrix is desired. Li et al. (2020) prepared PLA/TiO_2 nanocomposite films by casting process without the self-aggregation of TiO_2 nanoparticles. PLA was dissolved in dichloromethane at a solvent ratio of 4% (w/v) and the different amount of 0.2–1.0 wt% TiO_2 precursor obtained by sol-gel synthesis was added. The tensile strength, thermal stability, as well as the photocatalytic activity and antibacterial activities against *Staphylococcus aureus* and *Escherichia coli* of PLA films were evidenced. Higher thermal stability of PHB/TiO_2 nanocomposite films obtained by casting method was also reported (Iulianelli et al. 2018). The good thermal stability was attributed to the strong interaction between the dispersed phase and the polymeric matrix leading to the reduced molecular mobility of metallic nanoparticles. Partial miscibility of PHB produced by Biocycle blended with low molar mass PCL through solution casting

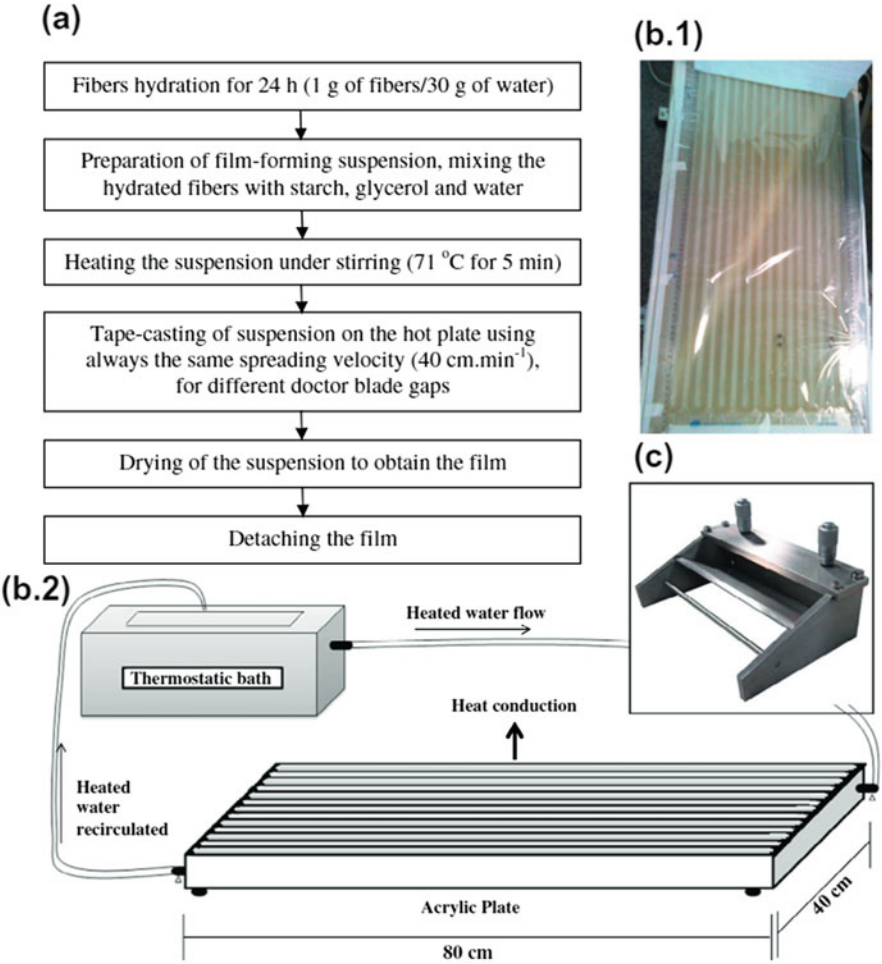


Fig. 4.4 (**a**) The preparation steps of cellulose fibers–starch films by tape-casting. (**b.1**) The support used for spreading suspension in the discontinuous (manual) tape-casting device. (**b.2**) Scheme of the acrylic plate. (**c**) Picture of a doctor blade device. (Reprinted with permission from de Moraes et al. 2013)

was reported by differential scanning calorimetry (DSC) and time-domain nuclear magnetic resonance (TD-NMR) (Cavalcante et al. 2017).

The casting technique is preferred for obtaining simple cassava starch films because those realized by extrusion processes present only satisfactory properties, due the high shear rates applied. Compared to the well-known casting technique used for the production of biodegradable and edible films, tape-casting is a suitable technology to scale-up the production of commercial starch-based films as it controls (by an adjustable blade at the bottom of the spreading device) the thickness of a suspension spread on large supports (de Moraes et al. 2013). Figure 4.4 shows the

Fig. 4.5 Large dimension film prepared by tape-casting, containing 3 g starch/100 g of suspension, 0.20 g glycerol/g dry starch, and 0.30 g fibers/g dry starch. (Reprinted with permission from de Moraes et al. 2013)

steps and the tape-casting device used by de Moraes et al. (2013) to realize films based on starch–glycerol–fiber suspensions with different amounts of cassava starch (Yoki Alimentos-Brazil) (3 and 5 g/100 g of suspension), glycerol (0.20 and 0.25 g/g of starch) and cellulose fibers (0 and 0.30 g/g of starch, 30 μm in diameter and 1.3 mm length). The flow properties (shear thinning) of the suspensions and interaction at the liquid–solid interface assure the film spreading by tape casting, the resulted uniform and easy to handle film is presented in Fig. 4.5. Starch-based films/coatings exhibit poor water vapor barrier properties and water solubility due to their hydrophilicity (Hassan et al. 2017).

4.2.7 Coating Method

PLA 3052D (NatureWorks) has been studied for the coating of industrial-grade Kraft paper abrasive products (Sundar et al. 2020). The coating parameters are a temperature of 30 °C, relative humidity of 75%, and coating time of 24 h in a vacuum oven, at a pressure of 100 mbar. The improvements in bursting strength of 6.3 kg/cm^2, air porosity of 245 s/100 cm^3, and surface roughness of 1.65 μm for paper coated were reported. Coating weight of 9 g/m^2 led to superior overall barrier properties for the fabrication of coated abrasive products. In another paper (Schlebrowski et al. 2019), a protective coating of PLA film with a thickness of 50 μm was achieved by the deposition of hydrogenated amorphous carbon, with the thickness layers in the range of 50 nm up to 500 nm, by plasma-enhanced chemical vapor deposition (PECVD) using acetylene.

Starch-based coatings have a great potential for food packaging application. Li et al. (2019) made a review comprising the recent developments in starch-based materials as coating binder and surface sizing agent, emphasizing the potential toxicity and migration of starch-based bionanocomposites for paper coating, as well as the environmental safety of their use. Starch is one of the preferred polymers to form edible coating films due to its availability, low cost, and good filmogenic capacity, forming colorless and tasteless films with high oxygen barrier capacity. It also presents good carrying properties for different actives which can protect fruits and vegetables from microbial decay or physiological disorders. Different compounds, such as plasticizers, surfactants, lipids, or other polymers, have been incorporated to improve the functional properties of starch-based films/coatings, such as water sensitivity, limited water vapor barrier properties, and mechanical resistance (Sapper and Chiralt 2018). Camatari et al. (2018) developed edible coatings based on cassava starch (Dinha Bá brand, Brasil) (0%, 0.25%, and 0.5%) and chitosan (0%, 0.25%, and 0.5%), and verified their effect on the physicochemical characteristics and post-harvest shelf life of the mango of "Tommy Atkins" variety. The most favorable coating contained 0.25% of chitosan and 0.5% of cassava starch and showed the lower rates of CO_2 production, improving with 3 days the mango's post-harvest shelf life compared with control fruits by decreasing the rate of the respiratory processes of mango, without compromising the proper ripening of the fruit.

The incorporation of lipid compounds, shellac (Sh) and oleic acid (OA), into Canadian non-GMO (non-genetically modified organism) yellow pea starch (PS) (Yantai Shuangta Food Co., China)—guar gum (GG) coatings and a layer-by-layer (LBL) approach reduced orange's respiration rate, ethylene production, weight and firmness loss, peel pitting, and orange's decay rate index (Saberi et al. 2018). For coating preparation, a first step 1 day before use was to dissolve 2.5 g PS, 0.3 g GG and 25% w/w glycerol in 100 mL degassed deionized water, heating the solution at 90 °C for 20 min in constant stirring, then cooling to room temperature. Second, the PSGG-Sh mixture was prepared by adding emulsifier OA (1% of the dry weight of PS and GG) and surfactant Tween20 (0.3 mL) to the PSGG solution, then food grade alcohol-based solution of shellac at 40% was added to the PSGG-OA-Tween 20-glycerol mixture. The final emulsion was gelatinized at 90 °C for 20 min on a hot plate with continuous stirring. After melting the lipids, samples were homogenized for 4 min at 22,000 rpm using a T25 Ultra-Turrax. The homogeneous film solution was cooled to room temperature with slow magnetic stirring, showing stability, with no phase separation, on the second day when the whole orange surface was sprayed with each coating solution uniformly by using a paint sprayer. Five treatments were applied on oranges: (a) PSGG; (b) PSGG-Sh; (c) bilayer formulation of PSGG as an inner layer with Sh solution as an external layer (PSGG/Sh); (d) CW (commercial wax, shellac-based "Citrus Gleam") and (e) distilled water acting as a control.

4.3 Recyclability of Bioplastics

The bioplastic compounds include, generally, bio-derived polyesters or polysaccharides highly sensitive to hydrolytic degradation at high temperatures. Soroudi and Jakubowicz (2013) evaluated the effect of PLA blends recyclability on the properties, in order to reduce the negative environmental impact of PLA waste. It was found that the mechanical properties of PLA did not deteriorate at its reprocessing by extrusion up to ten times. Another study performed by Chaitanya et al. (2019) showed that the high-performance PLA biopolymer (grade Ingeo 3260HP, acquired in the form of pellets from NatureWorks LLC, USA)/sisal fiber composite, was recycled (eight times) using the extrusion process. The screw speed of the extruder was set at 60 rpm, and the temperature profile from feed zone to die was 150/175/180/185 °C. Items with acceptable strength for nonstructural applications could be obtained during the first three recycled cycles. At eight recycled cycles, the decline of glass transition temperature with 10%, mechanical properties together with the hydrolytic degradation of PLA were observed. In a study of Lin et al. (2020), in view of overcoming the pollution and reutilization problem of the waste starch during the recycling process of old corrugated cardboard paper, hydrogen peroxide (H_2O_2) was used for ionization of waste starch, so improved mechanical properties of OCC paper were achieved during the reutilization. The steps for waste starch recycling include dissolving, ionization modification, and retention on the surface of the paper sheet. Cationic polyacrylamide improved the absorption of ionic starch onto the fibers, reducing the dissolution of starch in water in the re-pulping process. The retention rate of the modified starch was 30.2% higher than waste starch. Bioplastics based on starch reduces the carbon footprint of plastic packaging and also present some challenges regarding their recyclability. These modern starch materials are not recycled in the traditional way, but are compostable in controlled facilities, breaking down into carbon dioxide and water.

4.4 Conclusion

The research interest in the processing of commercial bioplastics, bio-based and/or biodegradable, has been recently increased. The typical technologies currently used to manufacture commercial bioplastics include injection molding, thermoforming, extrusion, and casting. The processing of most bioplastics takes place at a laboratory scale, using the conventional equipments for petroleum-derived polymers. However, new technologies such as 3D printing and electrospinning are developed for the manufacturing of prototypes with special properties for applications in the medical field, food packaging, automotive, and electronics areas. Optimization of the bioplastic compositions by adding renewable and inexpensive resources to facilitate both the reduction of cost and the maintenance or improvement of some properties still remains a challenge for use of bioplastics at an industrial scale.

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5 Protein-Based Bioplastics from Biowastes: Sources, Processing, Properties and Applications

Estefanía Álvarez-Castillo, Carlos Bengoechea, Manuel Felix, and Antonio Guerrero

Abstract

Even though proteins have been used for the development of plastic materials for a long time, their use has not proliferated when compared to other plant materials, such as starch or cellulose. Moreover, the current trend in the bioplastic market is based on polylactic acid or polyhydroxycarbonates, such that a feasible and globally accepted formulation of protein-based bioplastics is still pursued. The physical instability along time, together with the need of using non-food resources are drawbacks for the development of protein-based materials. Since lots of biowastes from the industry contain a considerable amount of proteins, the application of these natural polymers in the production of biodegradable materials would seem to benefit from a revalorisation of those resources, in agreement with a circular economy. This review is aiming for a comprehensive analysis of the current status of protein-based bioplastics, considering the main plant and animal sources that have been used, describing different wet and dry processing techniques and conditions, as well as the most reported properties (thermal, mechanical, optical, among others).

Keywords

Bioplastic · Protein · Biowaste · Characterisation · Food

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5.1 Introduction

The amazing versatility of polymers, together with their low price and density, has allowed their massive use in several fields (e.g. packaging, construction) since the middle of the twentieth century. However, all those years of uncontrolled disposal and the intrinsic negligible degradability have resulted in serious environmental issues, such as their accumulation which gives rise to the so-called Great Pacific garbage patch. In 2018, the world's plastic production totalled about 359 Mt (Plastics Europe 2019), from which approximately half is destined for the production of single-use items, mostly ending up in landfills, incinerated or flowing into the oceans (Hopewell et al. 2009). Around 75% of the cumulative global plastic production (~9.3 Gt) has been transformed into plastic waste, from which only around 21% has been recycled or incinerated (Karan et al. 2019). As the world population is becoming progressively more committed to environmental awareness, waiting for harder regulations to be developed, different solutions have been already approached in order to alleviate the burden caused by decades of misinformation and poorly eco-designed items. In this sense, the demand for bioplastics (i.e., plastic materials that are either biobased, biodegradable, or feature both properties) is currently around 1% of the annual plastic production and keeps rising. Moreover, it is a market whose production, according to the latest market data compiled by European Bioplastics, is forecasted to grow continuously (expecting to be ~3.2% higher every year), predicting 2.4 Mt for 2024 (European Bioplastics 2019).

Although the cost of bioplastics is currently higher than traditional plastics, a price reduction would be expected as they are produced at a larger scale (Mozaffari and Atefeh Kholdebarin 2019). Polylactic acid (PLA) and polyhydroxyalkanoates (PHAs) are found among the most promising alternatives to common plastics, together with starch and cellulose derivatives. Still, it should be born in mind that their prices are higher (PLA, >1.80 €/kg; PHA, 4.00–5.00 €/kg; thermoplastic starch, >2.50 €/kg; cellulosic plastics, 4.00–5.00 €/kg) than most consumed commodity polymers (polypropylene, 1.00–1.20 €/kg; polyethylene, 1.25–1.45 €/kg) (Molenveld and Bos 2019). However, end-users are increasingly willing to pay a higher price for so-called eco-friendly products, such as bioplastics. However, it can be noticed that the spread of PLA in several applications (packaging, 3D-printing) could become a drawback in the long term, considering its lack of complete biodegradability when mixed in aqueous media. Moreover, PLA requires months at high temperatures (~58 °C) under industrial composting conditions to fully degrade (Jem and Tan 2020). Instead, the production capacity for PHAs is estimated to more than triple in the next 5 years (European Bioplastics 2019), although the large-scale production of PHA still needs to overcome certain restrictions due to bacterial requirements. On the other hand, it should be highlighted that an important amount of food by-products, typically containing starch (~30–60 wt%), lipids (10–40 wt%), and protein (5–10 wt%), is wasted every year (i.e., one-third of the food produced worldwide) (Pleissner and Lin 2013; Hicks and Verbeek 2016). In this sense, starch, cellulose (and its derivatives), and proteins from food waste could be better used in the production of bioplastics, which would result in the

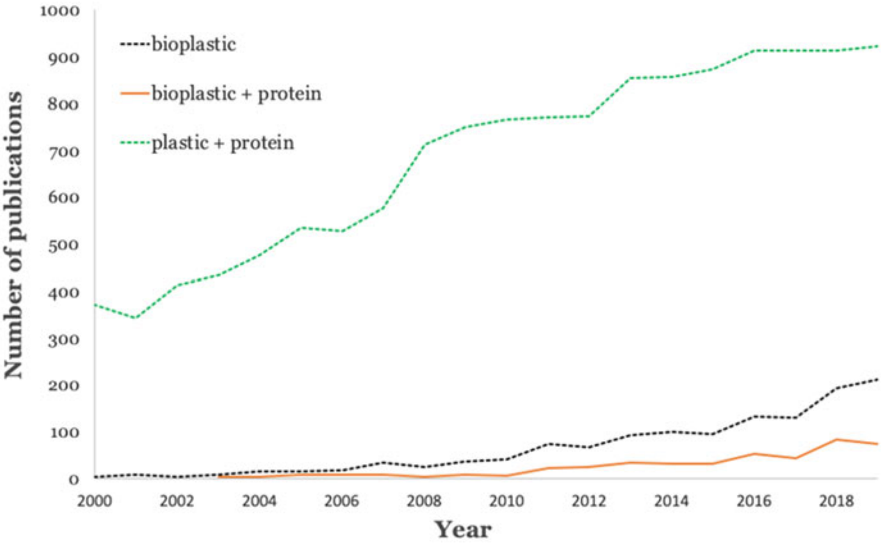

Fig. 5.1 Number of publications per year of protein-based bioplastics. (Source: Scopus database)

revalorisation of food by-products in an attempt for a circular economy. Moreover, such a revalorisation would avoid the expected rise in food prices and deforestation that might take place if human resources were deviated to produce plant-derived bioplastics (Fernández-d'Arlas 2019).

Quite a lot of starch-based plastics are already commercially available, conveniently blended with compostable plastics (e.g. PLA, PHAs, polycaprolactone (PCL)), and being used in applications such as bags, transport packaging, or service ware (e.g. cups, plates) (Gadhave et al. 2018). Cellulose-based plastics were among the first polymers used in the industry (i.e., celluloid, cellophane), and today, it is used as a matrix for biodegradable polymer composites, improving their mechanical properties and hydrophobicity. They find applications in different areas, such as ophthalmic, toys, sports goods, or spectacle frames (Gilbert 2017). The beginning of the industrial history of plastics were protein-based plastics (milk casein, zein, soybean, animal blood), commonly cross-linked with formaldehyde. Although the boost of plastics derived from petrochemicals and the competitiveness of the food industry have limited their large-scale development, a growing number of research studies have been focused on the feasibility of protein-based bioplastic (Fig. 5.1). Regarding their price, plant proteins are generally cheaper (0.5–1 €/kg) than those obtained from animals (2–10 €/kg). In any case, proteins derived from soy, sunflower, milk, whey, feather, or fish wastes have already been used in the production of bioplastics (Tsang et al. 2019). The use of proteins in the production of plastics shows the advantage of their lower processing costs, related to lower processing times and temperatures when compared to common polymers such as high-density polyethylene (HDPE) (Jerez et al. 2007a). However, their heterogeneity regarding the composition, which largely depends on the source, as well as their tendency onto gradual deformation and creep, makes it necessary to examine proteins in greater detail when considering their use in plastic production (Dorigato and Pegoretti 2012).

The fact that proteins are heteropolymers consisting of amino acids confers them a great versatility due to the great number of amino acidic compositions and spatial arrangements which may be found in the different sources. Thus, their chemical reactivity differs with respect to their amino acids position, nature and/or energy. In this sense, a relatively large amount of proline and hydroxyproline residues may promote a disruptive effect on typical protein secondary structures, resulting in a less ordered microstructure, which would favour a behaviour slightly more like that of a synthetic polymer throughout the processing stage (Bengoechea et al. 2007). The presence of polar aspartic and glutamic residues in the protein composition imparts a greater hydrophilic character to the protein. Additionally, the presence of cysteine residues is directly related to the potential formation of disulphide bonds along with the plastic processing, which induce high-molecular-weight aggregates that strengthen the material. Lysine residues may also interact and similarly lead to protein aggregates, such as they do in collagen-rich tissues, but they have also been used for protein functionalisation (Hwang and Damodaran 1996; Guilbert et al. 2006; Cuadri et al. 2017, 2018; Capezza et al. 2019, 2020a). In this case, the controlled reaction among a lysine-rich protein (e.g. soybean) and an anhydride (e.g. succinic) may be used to introduce carboxylic groups into the protein chain for increasing the polarity of the protein. The amino or imino group of lysine or arginine residues may also react with formaldehyde under moderate conditions, producing the corresponding methylol derivatives and forming then the methylene cross-linking (i.e., $N–CH_2–N$) (Klockenbusch et al. 2012). Cysteine, serine, or tyrosine residues have been used in different click-chemistry protocols in order to introduce alkynes in the polymeric chain (Li et al. 2008; Temming et al. 2013; Zhang et al. 2014).

It should be noted that much of the importance of bioplastics lies in their promotion of circular economy, as they use alternative feedstock's and offer a wider scope of end-of-life options for plastic products. In this sense, Karan et al. (2019) have highlighted the role of bioplastics either as biodegradability standards or as carbon sinks. In the former case, protein-based bioplastics have proven biodegradability either in water, soil or industrial compost (Spence et al. 1996). Particularly, in the case of plant protein-based plastics, lower greenhouse gas (GHG) emissions are expected than for petrochemicals, which has been associated to the absorption of carbon dioxide, CO_2, by the plants, returning to the atmosphere once the material is degraded (net zero carbon balance). For example, gluten bioplastics have displayed much lower values for energy consumption or GHG emissions than PLA or starch-based bioplastics (Guilbert et al. 2006). Moreover, it must be considered that stages like cultivation or drying have been reported to cause important environmental impacts during plant production. However, the use of renewable energies could help to alleviate that impact, achieving a low environmental single score impact when gluten films are extruded conventionally and finally incinerated at the end of the product life, with the resulting energy recovery (Deng et al. 2013). On

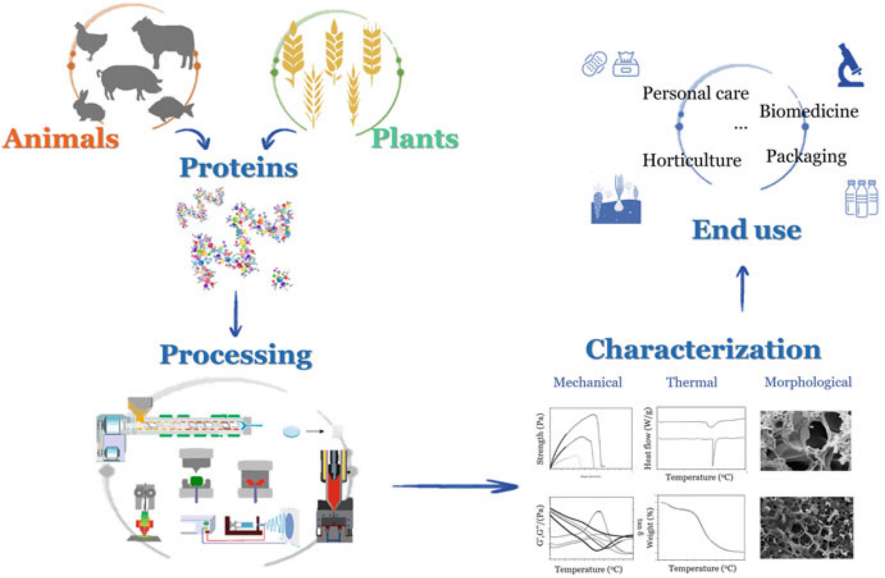

Fig. 5.2 Overview for the manufacture, characterization, and lifetime of protein-based bioplastics

the other hand, the production of animal protein-based foods typically show a greater environmental impact than plant-based foods (Ranganathan et al. 2016), with higher GHG emissions. However, fossil fuel-based energy is responsible for about 80% of the overall GHG emissions as compared to about 6% of animal agriculture (Pitesky et al. 2009). Moreover, the utilisation of food wastes (e.g. protein-rich flours obtained from oil-seed extraction, blood from slaughterhouses) for bioplastics production is a renewable sustainable process, being mostly biodegradable and compostable, meeting the criteria of international standards (Tsang et al. 2019). It should be highlighted that most proteins included in this review are co- or by-products from the food and biodiesel industries rather than wastes, not finding many studies related to the protein production from food waste, despite their high protein content (Aggelopoulos et al. 2014; Teigiserova et al. 2019).

The present book chapter deals with the main animal and plant biowastes that are being used in the production of bioplastics through different processing techniques, as illustrated in Fig. 5.2, that are characterized aiming for different applications (from the development of personal care products or packaging materials to biomedical or agricultural applications).

5.2 Protein Sources

Proteins from plants have a wide range of applications, mainly due to their availability and easiness to handle. On the other hand, the use of animal proteins has found more restrictions since they have been commonly rejected in cosmetic or food packaging products. Hence, they have been better used in agricultural applications. The following section summarises the main plant and animal protein sources from biowastes which are used in the field of bioplastic materials.

5.2.1 Plant Proteins

5.2.1.1 Soy Protein

Soybean possesses a high protein content (38–45%) and has a global production of around 350 Mt in 2018 (Food and Agriculture Organization of the United Nations 2018), being around 70% harvested in the United States, Brazil, and Argentina. A defatted protein-rich meal is obtained as a by-product of the soy oil industry, which is commonly used as low-price material for feeding animals and mostly discarded as industrial waste throughout the world (Yamada et al. 2020). Proteins from soybean mostly consist of globulins, constituting the major fraction by glycinin (7S) and β-conglycinin (11S). The ability of soy protein to form plastic materials has been used for a long time in Asian countries for the production of edible films known as yuba or tofu skin, which is obtained during the boiling of soy milk (Cuq et al. 1998; Zang et al. 2015). However, most research has focused on the processing of soy protein concentrates or isolates through casting, compression, or injection moulding. Soy protein bioplastics have generally displayed adequate mechanical properties, but they have been criticized mainly due to their generally low water-resistance, which is related to their high content in polar amino acid residues (aspartic, glutamic). Notwithstanding, this fact has been exploited in the field of superabsorbent materials (Hwang and Damodaran 1996; Álvarez-Castillo et al. 2018; Cuadri et al. 2018; Fernández-Espada et al. 2019).

5.2.1.2 Wheat Protein

Wheat gluten is a natural protein derived from wheat or wheat flour, obtained as a by-product from the bio-ethanol industry which possesses a high protein content (75–80%) (Ye et al. 2006). Wheat has a global production higher than 730 Mt, being China the greatest producer (~18% of the global production). Gluten is particularly rich in readily available glutamine (36%, compared to 17–20% in soy protein) and also in sulphur amino acids and enough branched-chain amino acids (i.e., leucine, isoleucine, valine) but poor in lysine (Flambeau et al. 2017). Although injection moulding processing has been quite limited due to its high molecular weight and aggregated structure (Cho et al. 2011), wheat gluten-based plastics have been obtained mostly in the presence of disruptive agents through casting or extrusion (John et al. 1998; Jerez et al. 2005; Chantapet et al. 2013; Jiménez-Rosado et al. 2019). Materials obtained from gluten are generally not water-soluble and present good barrier properties for oxygen and carbon dioxide, allowing a great number of non-food applications, such as films, plastics, and adhesives, related to its adhesiveness and cohesiveness, as well as its elastic properties (Day 2011; Capezza et al. 2020b).

5.2.1.3 Corn Protein

Zein is obtained as a by-product of the production of ethanol, starch, and oil from corn, from which it is the main protein (~45–50%). Zein may not be used directly for human consumption due to its negative nitrogen balance and its poor solubility in water, and therefore is mainly used as animal feed (Shukla and Cheryan 2001). Current zein manufacture is around 500 t/year from corn gluten meal. Zein is a prolamine, with a high content of hydrophobic amino acids (i.e., proline, glutamine, asparagines) (Elzoghby et al. 2012). Like most plant proteins, it is insoluble in water but soluble in alcohol. Due to its film-forming ability which also provides a moisture barrier, zein has been widely used in the food and packaging industry, as a grease-proof coating for several products (Elzoghby et al. 2015). Thus, the water vapour barrier properties of polypropylene cast films improved with zein coating (Tihminlioglu et al. 2010). Also, the incorporation of cross-linking agents or silicate complexes in zein-based materials increased their tensile strength, hindering their gas permeability (Shukla and Cheryan 2001). Moreover, it has been used in injection moulded starch-based plastics due to its plasticising effect, although special attention should be put on the brittleness they display when using unmodified starch under dry conditions (Lim and Jane 1994). Several studies have been focused on the extrusion of zein through slit dies to form films, observing that the addition of oleic acid promoted elongation and diminished their tensile strength (Santosa and Padua 1999; Ha and Padua 2001; Herald et al. 2002).

5.2.1.4 Animal Proteins

Keratin

Keratin is a strong natural polymer that may be extracted from epidermal appendages such as nails, claws, beak, hair, horns, and, especially, feathers or wool (~90%). In spite of its potential, 5 Mt of chicken feathers alone are discarded per year (Iqbal et al. 2017). Keratin is actually a mixture of high-molecular-weight proteins that may be obtained from appendages by extraction through chemical, enzymatic, or ionic solution methodologies, giving rise to properties that are highly affected by the extraction procedure (Fernández-d'Arlas 2019). It is composed of fibrous proteins with a high presence of cystine (Barone and Schmidt 2006; Shi and Dumont 2014b; Tesfaye et al. 2017; Ramakrishnan et al. 2018), being subdivided into two different classes of secondary structures: α- and β-keratin (Shah et al. 2019). Keratin has been processed mainly through casting (Ramakrishnan et al. 2018; Fernández-d'Arlas 2019), resulting in transparent materials, with proper UV barrier properties, thermal stability from 50 to 200 °C, and water sensitivity. Regarding their mechanical properties, they have been reported to possess high intrinsic toughness when cross-linked (Fernández-d'Arlas 2019). It has also been used as an additive for synthetic elastomers, giving rise to materials with good thermal, mechanical, flame resistant, and thermo-oxidative properties (Garrison et al. 2016).

Milk Proteins

Caseins account for around 80% of milk proteins, possessing an aminoacidic composition highly dependent on the source (Thorn et al. 2014). Around 852 Mt of milk were produced in 2019 from different sources (81% cow milk, 15% buffalo milk, and 4% for goat, sheep, and camel milk) (OECD and Food and Agriculture Organization of the United Nations 2020). In bovine milk, casein is the main protein (~25 g/L) and is principally based on α_{s1}-casein (38%), α_{s2}-casein (10%), β-casein (36%), and κ-casein (13%) (Audic et al. 2003). Milk proteins are widely used in the food industry (e.g. beverages, formulated meat products, margarine, whipped foods, desserts, puffed snacks, processed cheese) due to their surface-active properties (Kelly et al. 2009). However, the cleaning of dairy factory plants results in abundant casein-rich water, which could be revalorized as a source of protein material for the development of bioplastics (Ryder et al. 2020). Thus, transparent, flexible, and tasteless films can be formed from casein, which water resistance may be improved through cross-linking (Cuq et al. 1998; Chambi and Grosso 2006).

Whey proteins, which account for around 5.4 g/L in bovine milk (Audic et al. 2003), are a by-product of cheese processing and are those which remain in solution after removal of the caseins from milk, being β-lactoglobulin the main protein (about one-half of the total protein present) (Zadow 2003). Bioplastics produced from whey are not very different from those prepared from caseins, being possible to achieve insoluble films partly due to disulphide covalent bonding (Cuq et al. 1998). Composites including whey and egg albumin proteins have been prepared through compression moulding, behaving similar to zein-based bioplastics, which is more expensive than whey protein (Sharma and Luzinov 2013).

Egg Albumin

The egg albumin (also known as egg white) is found in eggs surrounding the yolk. It represents ~65% of the egg's weight, and it is made up of 88% water, 11% proteins, and 1% carbohydrates and minerals. Considering that around 77 Mt of hen eggs were obtained in 2018 (~92% of the global egg production) (Food and Agriculture Organization of the United Nations 2018), a production of 50 Mt of egg white could be estimated. Since the food industry separates many times the egg white from the yolk, it is considered a food by-product (Sharma et al. 2019). The qualification of egg albumin, mainly composed by ovalbumin (54%) (Stevens 1991), as biowaste remains uncertain, as it is well used by the food industry. However, even if it has some food applications (i.e. in the wine industry, bakery), there is a surplus (Sharif et al. 2018). It may be processed into highly transparent bioplastics, obtained though compression, extrusion, or injection moulding (Jerez et al. 2007b; Martinez et al. 2013; López-Castejón et al. 2016; Fernández-Espada et al. 2016a; Martín-Alfonso et al. 2018; Pranata et al. 2019), which could be well used to produce novel edible products (De Pilli 2020). Egg albumin-based plastics are clearer and more transparent than those based on wheat, soy, or corn proteins and may find application not only in the food industry but also in the pharmaceutical industry as water-soluble bags meant for conditioning and protecting additive doses (Cuq et al. 1998). Egg white-based bioplastics have proven to be adequate for rigid food packaging, which shows an efficient barrier to oxygen but not to water (Pranata et al. 2019).

Blood

Blood is a certain by-product of the meat industry (~4500 ML in 2016) representing up to 4% of the live animal weight and, as only 30% is used by the food industry, is being extensively discarded into municipal sewers and landfills (Gatnau et al. 2001; Sanders 2018). Blood is composed of water, cells, enzymes, proteins, and other organic and inorganic substances that can be separated into two fractions: the cellular fraction (30–40 wt%) and plasma (up to 60 wt%) (Bah et al. 2013). After processing blood into those fractions, different applications have been found, such as a water-holding agent in products like frankfurters (Hurtado et al. 2011, 2012) or as a replacer of other ingredients like egg albumen (Raeker and Johnson 1995). It contains typically around 18% of protein (Peters 1975), which may be used in other non-food related applications, like packaging (Nuthong et al. 2009) or super-absorbent materials (Álvarez-Castillo et al. 2019). Particularly, some authors have performed extensive research on bioplastics based on a commercial blood meal (Novatein) obtained through extrusion or injection moulding (Verbeek and van den Berg 2011; Adamy and Verbeek 2013; Verbeek et al. 2017). Plasma obtained after blood centrifugation and drying contain around 70% of protein (Howell and Lawrie 1983), which is mainly composed of albumin (50–60%), α-, β-, and γ-globulins (40–50%), and fibrinogen (1–3%) (Dàvila et al. 2007). Recently, the potential of the plasma fraction in the field of injection moulded superabsorbent materials has been highlighted (Álvarez-Castillo et al. 2019, 2020a, b).

Collagen and Gelatine

Collagen, which may be found within the stromal fraction of meat proteins, is the most abundant protein in mammals. It accounts for 30% of the whole-body protein content, and it is mainly extracted from pigskin (46%), bovine hide (29%), and pork and cattle bones (23%) (Mullen et al. 2015). The collagen macromolecule forms a triple helix that is abundant in proline and hydroxyproline residues (Noorzai et al. 2019). When collagen is partially hydrolysed, it results in the production of gelatine (500 kt/year) (Lestari et al. 2019), which has been successfully and widely employed to create packaging films as it is relatively cheap and abundant (Lacroix et al. 2014; Chentir et al. 2019). Edible films and packaging have been produced from gelatine due to its thermo-reversible properties, a melting point close to that of the human body temperature, its ability to be used as a functional agent carrier, its good oxygen barrier properties, abundance occurrence, biodegradability, and excellent ability to form films (Murrieta-Martínez et al. 2018; Al-Tayyar et al. 2020). It has been processed through casting, extrusion, or electrospinning, for food or biomedical applications. Gelatine films are transparent, flexible, water-resistant, and impermeable to oxygen (Cuq et al. 1998).

5.2.2 Processing of Protein-Based Bioplastics

Nowadays, conventional plastics are among the most common materials used for the manufacture of all kind of items. However, the wastes generated after the service life of these oil-derivative materials aim to promote a change towards the use of eco-friendlier alternatives such as bioplastics (Peelman et al. 2013). This recent trend forces redesigning the processing techniques typically used (e.g. injection moulding, extrusion, compression moulding), since these new materials show different properties (Ashter 2016). Optimum processing parameters must be selected for bioplastic processing, and as these parameters are highly dependent on the final product and its end-use, they differ from those used for traditional polymers (Lukubira and Ogale 2013). In these terms, many techniques have been employed in the manufacture of protein-based bioplastics, where injection moulding, extrusion, casting, compression moulding, electrospinning, and three-dimensional (3D) printing are especially relevant.

The processing of raw materials for the manufacture of protein-based bioplastics requires either their solution in different solvents, in wet techniques, like casting or electrospinning, or the use of plasticizers, in dry techniques, such as compression moulding, extrusion, or injection moulding. Plasticizers are low molecular-weight components which interact with protein chains, reducing protein-protein interactions. Their effect is based on changes in ionic interactions, covalent links, or hydrogen bonds during the processing stages (Krochta and Hernández-Izquierdo 2008; Gómez-Estaca et al. 2016), enabling the plasticisation of materials (Budhavaram et al. 2010). As a result, plasticizers ease protein-based bioplastic processing and increase the flexibility of the final product. These improvements in material processing could be also accomplished by heating the protein-containing raw material above the glass transition temperature (T_g). However, lower-plasticised proteins have a T_g higher than the protein degradation temperature. In bioplastic production, glycerol, sucrose, sorbitol, and polyethylene glycol are among the most commonly used plasticizers (di Gioia and Guilbert 1999). For bioplastic processing, homogeneous protein-based solutions or blends are required, for which a thorough mixing stage of the protein system with additives (e.g. plasticizers) or other ingredients is commonly performed before further processing (e.g. casting or injection moulding) or during the process itself (e.g. extrusion) (Redl et al. 1999; Cortés-Triviño and Martínez 2018). For the former case, different mixing methods such as hand-mixing (Martin 1948), melting a mixture of protein and fatty acids (Pommet et al. 2003b), the use of a roller pin (Lai and Padua 1997), and a torque and temperature controlling mixing rheometer have been used (Perez et al. 2016; Álvarez-Castillo et al. 2019; Fernández-Espada et al. 2019). In this section, different manufacturing techniques of protein-based bioplastics are discussed, pointing out the key parameters typically required for every technique (Fig. 5.3).

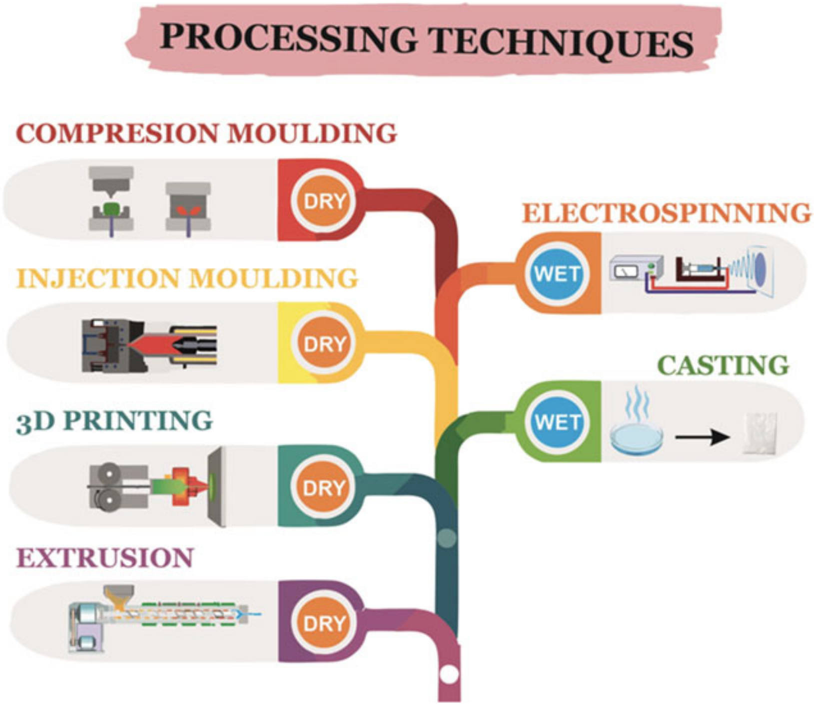

Fig. 5.3 Processing techniques typically used for protein-based bioplastic either by dry or wet processing

5.2.2.1 Wet Techniques

Casting

Casting has been the most used physicochemical processing method for protein-based bioplastic films. This wet processing method consists of three consecutive steps: (1) first, a chemical reagent is used to disrupt disulphide bonds between protein chains (Bernard et al. 2018); (2) secondly, a multicomponent solution is prepared (the solution contains protein, plasticizer and solvent, although other minor components, such as cross-linkers or antimicrobials, may also be included); (3) lastly, the solution is spread and the solvent evaporates (or dries), giving rise to the desired protein-based bioplastic film (Guilbert et al. 2006; Gómez-Estaca et al. 2016). The basic control of this strategy depends on the concentration of solvent used, pH, temperature, and drying conditions (Guilbert et al. 2006).

The casting technique is mostly used at lab-scale, finding potential applications for coating materials in the packaging industry (Farris et al. 2009; Muriel-Galet et al. 2013; Gómez-Estaca et al. 2016). The hydrophilic character of proteins leads to biobased films with enhanced barrier properties compared with most commercial plastics based on petrochemicals (Krochta 1992; McHugh and Krochta 1994). Nevertheless, their mechanical properties are poorer than those of traditional films which are tougher and possess higher elongations (Krochta and Hernández-Izquierdo 2008; Wihodo and Moraru 2013). To overcome this drawback, the physical properties of protein films could be enhanced through various methods, like studying the plasticisation effect (Sothornvit and Krochta 2000a, b, 2001), modifying the pH value (Avena-Bustillos and Krochta 1993; Brandenburg et al. 1993), lipid addition (Avena-Bustillos and Krochta 1993; Sohail et al. 2006), or increasing cross-linking in the protein network (Martucci et al. 2006). On this basis, Gontard et al. (1994) highlighted that the pH at which the film-forming solution was prepared had a remarkable effect on the strength of gluten-based films (Gontard et al. 1992; Lim et al. 1992). This improvement in mechanical properties was related to changes in protein conformation with pH (Wihodo and Moraru 2013). This strategy has been developed in several studies, aiming the generation or the improvement of films from different protein sources, such as zein corn (Martin 1948; Gennadios and Weller 1990; Lai and Padua 1997; Ghanbarzodeh et al. 2007), soy (Rhim et al. 2000; Kim et al. 2002; Guerrero et al. 2010; Guerrero and de la Caba 2010; Bourny et al. 2017), milk (Avena-Bustillos and Krochta 1993; Sothornvit and Krochta 2000a, c; Sohail et al. 2006; Zhou et al. 2009), pea (Choi and Han 2001, 2002; Kowalczyk et al. 2014; Acquah et al. 2020), and fish (Cuq et al. 1996, 1997; Ahmad et al. 2015; Araújo et al. 2018; Bernard et al. 2018; Ansari et al. 2019). A wide literature has been set about this bioplastic processing technique, being gluten the most recurrent protein used since this protein contains a high number of cysteine residues (Gontard et al. 1992, 1993, 1994; Lim et al. 1992; Gennadios et al. 1993; Gontard and Guilbert 1994; Guilbert et al. 1995; Heralp et al. 1995; Cuq et al. 1996; Micard et al. 2001; Mangavel et al. 2002; Hernández-Muñoz et al. 2003, 2004; Kayserilioğlu et al. 2003; Pommet et al. 2003b; Tunc et al. 2007; Lagrain et al. 2010; Balaguer et al. 2011b). This high number of cysteine residues can be considered exceptional in the particular case of gluten since not many oilseed plants possess them (Newson 2012). These residues are key for the development of protein-based bioplastic since they promote covalent bonds between protein chains, facilitating the formation of a network through simple heating or solution casting (Lagrain et al. 2010).

Electrospinning

Electrospinning is a processing technique for polymers which result in nanofibrous materials after subjecting polymer solutions to a high-voltage electric field (Huang et al. 2004; Bhushani and Anandharamakrishnan 2014; Fabra et al. 2016). These materials have large specific surface areas, high porosity, small pore sizes, and low density (Mendes et al. 2017). The commonly used experimental set-up consists of a high voltage direct current (DC) source (typically from 5 to 25 kV) connected to a sharp needle and a collector. The flow thorough the needle is controlled by a syringe pump, whereas the liquid goes from the needle to the collector boost by the driving force of an electric field. The typical distances between the needle and the collector are between 10 and 20 cm (Ramakrishna 2005; Wang and Hsiao 2016). The effects produced by the most important variables of the electrospinning process on the morphology of the fibres formed has been recently summarized (Perez-Puyana et al. 2020b). Moreover, a variant of this method is the use of a rotating collector to increase fibre alignment (required for some applications such as biomaterials) (Pan et al. 2006; Teo and Ramakrishna 2006; Aviss et al. 2010). When a polymer solution is subjected to an electric field, it elongates to some extent from the needle tip, forming the so-called Taylor's cone (Schiffman and Schauer 2008). The morphology of the fibres eventually formed depends on pH, electrical conductivity, interfacial tension, and viscosity of the biopolymer solution (Kriegel et al. 2008). Although this is the most common experimental set-up (syringe-needle-collector), other authors have used needleless roller electrospinning, which consists of a semi-submerged rotating roller in a polymer solution. The dispersion is also charged (higher voltages, up to 60 kV), driving the fibres towards the collector (Kutzli et al. 2018, 2019).

Like other processing techniques, electrospinning was initially used for synthetic polymers; however, it has been also used for biopolymer systems (e.g. proteins, polysaccharides, or their mixtures with other polymers). Generally, proteins are not considered to be easily spinnable. Their secondary and tertiary conformations give them complex structures and weak internal interactions, which hinder fibre formation (Nieuwland et al. 2013; Sullivan et al. 2014). Some authors have proposed that proteins must be unfolded to some extent before electrospinning (Dror et al. 2008). To achieve this, the addition of disrupting agents (i.e. sodium dodecyl sulphate and urea), the use of specific solvents, and heat application have been proposed (Wongsasulak et al. 2007; Regev et al. 2010; Mendes et al. 2017). In this sense, different water/polar solvents such as 2,2,2-trifluoroethanol (TFE) (Dror et al. 2008), hexafluoroisopropanol (HFIP) (Lang et al. 2013), 2,2,2-trifluoroethanol (Huang et al. 2004), trifluoroacetic acid (TFA) (Huang et al. 2015), dimethyl sulfoxide (DMSO) (Elamparithi et al. 2016), and acetic acid (Wang and Chen 2012) were used for the development of protein-based nanofibrous mats. Thus, nanofibrous composite mats based on gelatine/PCL (Gautam et al. 2013; Perez-Puyana et al. 2020a) or gelatine/PVA mixed solutions (Perez-Puyana et al. 2018) have been developed for scaffolds with potential applications in tissue engineering. A similar approach has been used by other authors for several applications such as food packaging or drug-delivery (Li et al. 2005; Zeng et al. 2005; Fabra et al. 2013; Bhushani and Anandharamakrishnan 2014; Hu et al. 2014). Moreover, protein/carbohydrates (conjugated or not) have been also used for the development of nanofibrous mats. Recently, Aman Mohammadi et al. (2019) produced food-grade mats using WPI and guar gum for the generation of nanofibrous mats. According to Yoon and Kim (2008), protein electrospinnability can be improved by using polysaccharides, although this strategy is not very usual. Different protein sources have already been used for the development of nanofibrous mats such as BSA (Dror et al. 2008; Regev et al. 2010), gelatine (Huang et al. 2004, 2015; Gautam et al. 2013; Elamparithi et al. 2016), silk fibroin (Lang et al. 2013), soy (Song et al. 2011), egg albumen (Wongsasulak et al. 2010), or whey protein (Aman Mohammadi et al. 2019).

5.2.2.2 Dry Techniques

Compression Moulding

The first processing strategy for polymers with industrial applications was compression moulding, being used mainly for thermosetting polymers since the beginning of the twentieth century for manufacturing plastic products (Tatara 2011). However, it can be also used in thermoplastic polymers processing (Åkermo and Åström 2000; Trende et al. 2000). Compression moulding consists of the application of a large pressure into a closed mould which contains the polymer before curing (in the case of thermosetting polymers) or the melt polymer (in the case of thermoplastic polymers). When protein-based bioplastics are processed by compression moulding, the dough-like material obtained after convenient mixing is placed on the mould cavity (Tatara 2017). Contrary to injection moulding, both sides of the mould are compressed themselves, and the dough-like material for protein-based bioplastics does not flow through any cavity (Park and Lee 2012). Since a high flowability is not strictly necessary for this processing technique, compression moulding has been used for protein-based biocomposite materials reinforced with fibres (Otaigbe and Adams 1997; Tkaczyk et al. 2001; Huang et al. 2003; Deng et al. 2006; Ye et al. 2006; Shubhra et al. 2011; Sharma and Luzinov 2013; Saenghirunwattana et al. 2014; Das et al. 2019; Nilsuwan et al. 2020). However, one of its major disadvantages is that it is a batch process, which limits its industrial application (Tatara 2011). Despite this drawback, many proteins have been processed by compression moulding, such as corn (di Gioia and Guilbert 1999; di Gioia et al. 2000), soy (Shubhra et al. 2011), gluten (Jerez et al. 2005; Balaguer et al. 2011a; Martinez et al. 2013; Zárate-Ramírez et al. 2014a, b, 2011; Zubeldía et al. 2015), egg white (Jerez et al. 2007b), cottonseed (Yue et al. 2011, 2012) and whey, among others (De Graaf 2000; Gómez-Heincke et al. 2017). As for the processing conditions, the temperature of the mould should always be higher than the T_g of the dough-like protein blend studied, which means that not good results were generally found below 60 °C (Álvarez-Castillo et al. 2019). Different studies analysed the influence of temperature on protein-based bioplastics processed by compression moulding at a temperature lower than the degradation temperature of proteins which depends on the protein systems analysed. In this sense, temperatures around 100–120 °C were the highest temperature commonly used in different protein systems (di Gioia and Guilbert 1999; di Gioia et al. 2000; Zubeldía et al. 2015), although other authors increased the mould temperature up to ~180 °C (De Graaf 2000). As for moulding pressure, different mould pressures have been used, although they typically are ~10 bar, for a compression time of 2–10 min (De Graaf 2000; Yue et al. 2012; Gómez-Heincke et al. 2017).

Injection Moulding

This technique is broadly used in polymer manufacturing when a particular shape or a high-dimensional-precision material is required (Ashter 2016). Protein-based bioplastics obtained by injection moulding typically consists of two stages: first, the homogeneous blend is introduced into the thermoset injector feeding cylinder, which heats the sample conveniently; once it has been softened, it is forced to flow into the mould. Thus, blends go through a nozzle into the pre-heated mould cavity, adequately shaped (Schulze et al. 2017). Thus, cylinder and mould temperatures, injection pressure, and injection and post-injection times are among the most relevant parameters (Ashter 2016). Numerous publications have highlighted the influence of processing temperature of protein/glycerol blends not only on the dough-like material processing but also on the final properties of protein/based bioplastics (Felix et al. 2014; Perez et al. 2016; Fernández-Espada et al. 2016a; Álvarez-Castillo et al. 2019, 2020a). This technique has been employed in the development of several protein-based-bioplastics using different protein sources, such as soy (Vaz et al. 2003; Mohanty et al. 2005; Tummala et al. 2006; Cuadri et al. 2016, 2017, 2018; Fernández-Espada et al. 2016b, 2019; Bourny et al. 2017; Álvarez-Castillo et al. 2018; Tian et al. 2018), albumen (Jerez et al. 2007b; Fernández-Espada et al. 2016a), whey (Sharma and Luzinov 2013), porcine plasma (Álvarez-Castillo et al. 2019, 2020a, b), rice (Felix et al. 2016b), green pea (Perez-Puyana et al. 2016; Perez et al. 2016) or sunflower protein (Orliac et al. 2003).

Moulding temperature exerts a relevant effect on the final properties of protein-based bioplastics, while the effect of pressure has been reported to have a moderate effect (Perez-Puyana et al. 2016). The adequate selection of both mould and cylinder temperatures can improve the flow through the nozzle and facilitate the correct mould fill-up and protein reinforcement. However, some properties could be compromised. Specifically, when developing swelling materials, an increase in mould temperature commonly give rise to greater mechanical and viscoelastic properties, but the swelling is then limited (Xiaoqun et al. 1999). Higher temperatures cause a strengthening in the structure due to the formation of new interactions between protein chains; however, this reinforcement of the protein network makes the swelling of protein-based bioplastic specimens difficult (Álvarez-Castillo et al. 2018, 2019).

Extrusion

Extrusion is one of the most common techniques used for the production of plastics (Pommet et al. 2003a; Robertson 2016; Zink et al. 2016), being a continuous processing method which allows the control of temperature in a double-casing equipment inside which, at least one inner endless screw transports the protein-based mixture from the hopper to the extrusion nozzle or die. The movement of the endless screw subjects the sample to a high shear rate allowing the homogenisation of the protein source with the plasticizer without the need of a prior mixing stage. However, when a protein-based system is subjected to extrusion, the operating conditions to be applied are quite different from the typical extrusion of thermoplastic materials. The thermal response of protein/plasticizer blends is generally complex, combining regions of thermoplastic behaviour with regions where heat-induced cross-linking of different nature can be promoted (e.g. protein aggregation, disulphide bonds, Maillard reactions). These phenomena typically prevent melting, and instead, thermal degradation of protein takes place at relatively moderate temperatures. As a result, a previous thermal characterisation of the protein/

plasticizer blend is required, in order to define the temperature profile to be applied inside the extruder, in which the lowest applied temperature must exceed the T_g of the protein/glycerol blend (Ferry 1980), which depends on the percentage of plasticizer in the sample (Slade et al. 1993; Chantapet et al. 2013). The proper selection of plasticizer amount, shear rate, and temperature permits the formation of desired soft viscoelastic blends along the endless screw. Eventually, the material is shaped as desired when passing through the extruder die (Ullsten et al. 2009) and subsequently stabilized by cooling (Zink et al. 2016). An adequate selection of the temperature profile is key, as excessive early high temperatures in the screw may promote extensive cross-linking and aggregation before the sample reach the die (Verbeek and van den Berg 2010). Thus, the most influential parameters in protein-based bioplastics are shear impact, time, and temperature (co-dependent with specific mechanical energy) (Redl et al. 1999; Ullsten et al. 2009). Thus, Redl et al. (1999) highlighted that the appearance of a gluten/glycerol extrudate (i.e., smooth, disrupted) was greatly dependent on the operating conditions, obtaining better results when employing low specific mechanical energy and temperatures around 60 °C. In the same sense, Pietsch et al. (2017) indicated that the thermal treatment along the screw exerted the main influence on wheat gluten processing, while pressure or the specific mechanical energy did not possess a noticeable influence.

Generally, protein-based bioplastics need a lower temperature profile to be extruded than petrochemical polymers because proteins have a lower softening temperature (Pommet et al. 2003a; Verbeek and van den Berg 2010). Although the protein more widely processed through extrusion has been wheat gluten (Redl et al. 1999; Pommet et al. 2003a, 2005; Bengoechea et al. 2007; Ullsten et al. 2009, 2010; Chantapet et al. 2013; Jiménez-Rosado et al. 2019; Pietsch et al. 2019a, b), other proteins sources such as soy protein (Arêas 1992; Zhang et al. 2001; Liu et al. 2004, 2005, 2010; Felix et al. 2018) have also been used.

3D Printing

3D printing has brought the possibility of fabricating geometrically complex materials after the software design of the model without expensive equipment investments. Its main competitive disadvantage comes up when large production is required (Chia and Wu 2015). This technique is based on solid free-form fabrication (SFF), being applied in different ways such as stereolithography (SLA), digital light processing (DLP), fused deposition modelling (FDM), selective laser sintering (SLS), selective laser melting (SLM), or binder Jetting (BJ), among others (Jasveer and Jianbin 2018). 3D printing has been originally developed for synthetic polymers and has shown promising biomedical applications (Bose et al. 2013; Chia and Wu 2015). Schulze et al. (2017) indicated that 3D-printing was the process with the lowest energy demand per part for batch sizes when bioplastics were processed. Although the literature related to protein-based products is scarce, some studies have been published using proteins for the manufacture of 3D printed food products (Lille et al. 2018; Liu et al. 2018). This is the case of the studies about suitable printed samples from pea protein (Oyinloye and Yoon 2021) (with alginate), soy protein (Chen et al. 2019), plasma protein (Álvarez-Castillo et al. 2021), and milk proteins

(Liu et al. 2018, 2019) for which it was concluded that the rheology has a relevant role in the control of a proper process of 3D printing.

5.2.3 Characterisation of Protein-Based Bioplastics

Any manufactured material needs to satisfy different specifications in order to be suitable for a specific end-use application. Among these requirements, certain values for their mechanical, thermal, and/or optical properties may be desired, which advises their assessment through different experimental techniques. Analysis techniques make use of physical principles and properties for a systematic quantification of macroscopic parameters, whose analysis can be related to the microscopic (even molecular) structure of the materials evaluated. The main experimental techniques used for the characterisation of bioplastics are shown in this section divided into three blocks: mechanical, thermal, and morphological properties.

5.2.3.1 Mechanical Properties

The mechanical behaviour of a material is crucial when its deformation response to an applied stress is attempted to be understood. Complex materials typically exhibit viscoelastic behaviour when are subjected to deformation, which can be of different nature. The mechanical properties of bioplastics have been divided into two sections: rheological and tensile-strength tests.

Rheological Tests

Rheology is a well-established science applied for a wide range of materials (from liquids to solids) (Uchikawa 2001). Although rheology is defined as the “study of flow behaviour” and it was initially applied to liquid materials, the linear and non-linear viscoelasticity of solid materials can be analysed to obtain insight into characteristics of the material analysed (Barnes 2000). The viscoelastic behaviour can be analysed like the sum of pure elastic and viscous characters (Hookean and Newtonian behaviours, respectively). In rheology, this intermediate behaviour has been represented by the combination of spring and dashpot elements for elastic and viscous components, respectively (Banks et al. 2011). In this section, the rheological characterisation of bioplastics has been divided into oscillatory dynamic and continuous deformation tests.

Dynamic Mechanical Analysis (DMA)

DMA tests are of great importance in studying the behaviour of viscoelastic materials. The sinusoidal deformation of a viscoelastic material is the result of the application of sinusoidal stress (and vice versa). The analysis of the sinusoidal stress-strain test performed can be used to find the dynamic viscoelastic moduli (Barnes 2000). In the case of viscoelastic materials, there is a region (the so-called linear viscoelastic region) where the viscoelastic moduli do not depend on the deformation applied, and the stress is the sum of an in-phase response (elastic component, recoverable energy) and an out-of-phase response (viscous component, non-recoverable energy). According to this, the elastic (or storage) and the viscous (or loss) moduli (E' or G' and E'' or G'', respectively) can be defined as follows:

$$\text{Elastic modulus } (E' \text{ or } G') = \frac{\sigma}{\gamma}\cos(\delta) \tag{5.1}$$

$$\text{Viscous modulus } (E'' \text{ or } G'') = \frac{\sigma}{\gamma}\sin(\delta) \tag{5.2}$$

where δ is the retardation in-phase angle. To stay within this linear region, a small amplitude oscillatory stimulus (strain or stress) must be applied to obtain a small amplitude response (stress or strain). A strain (or stress) sweep test is typically carried out at constant temperature and frequency to delimit the linear viscoelastic range (i.e. obtaining the critical strain value). Then, the most common experiments are frequency sweep tests and temperature sweep tests (at strain lower than the critical value). This last type of test, which is also known as DMTA measurements, will be commented below with other techniques for determining thermal properties. In any case, linear viscoelastic tests can be carried out under different deformation modes, such as bending, either using single or dual cantilever geometries (Sun et al. 2008; Felix et al. 2016b) or three-point bending (Bruyninckx et al. 2015); compression with parallel plates (Gomez-Martinez et al. 2009); and torsion (Álvarez-Castillo et al. 2018).

Moreover, these experiments (e.g. small amplitude oscillatory compressional temperature sweep tests) can be applied to protein/plasticizer blends, which are to be processed into bioplastics, in order to select proper thermal processing conditions (Felix et al. 2015a; Álvarez-Castillo et al. 2019). Characterisation of bioplastics by frequency sweep tests under linear conditions has particular relevance since their results can be related to the internal structure of the material analysed (Ferry 1980; Jerez et al. 2005). For instance, Domenek et al. (2004) correlated the linear viscoelastic results of wheat gluten-based bioplastics from frequency sweep tests with their biodegradability response. The structural characterisation by means of frequency sweep tests was carried out for bioplastics of different protein sources such as rapeseed (Delgado et al. 2018), pea (Perez-Puyana et al. 2016; Perez et al. 2016), blood meal (Álvarez-Castillo et al. 2019), albumin (Fernandez-Espada et al. 2013; Felix et al. 2014), and zein (Ghanbarzadeh et al. 2006) amongst others (Felix et al. 2015b, 2016a). Martín-Alfonso et al. (2014) compared the elastic modulus of albumen-based bioplastics and commercial low-density polyethylene (LDPE) observing that E' for albumen-based bioplastics (around 3.5×10^9 Pa) was higher than for LDPE under the same experimental conditions (around 1×10^9 Pa). Moreover, these authors also stated that the thermoplastic character of LDPE involved a dramatic drop in E' at temperatures above 60 °C, which was not observed in albumen-based bioplastics.

Continuous Deformation Tests

Among the different continuous deformation tests that can be performed, stress relaxation, and creep tests can be highlighted. The former applies a constant strain, whereas the stress is monitored as a function of time until reaching a plateau value. In contrast, creep tests measure a progressive deformation under constant stress. Since deformation depends on the stress applied, compliance (J) can be defined as the ratio between the deformation (γ) and the stress (σ) applied:

$$J(t) = \frac{\gamma(t)}{\sigma}$$

Within the linear viscoelastic range, compliance remains unaltered, regardless of the stress applied. Creep tests were used to characterise the rheological properties of soy-based (Reddy et al. 2009) or fish-based films (Cuq et al. 1996), among others. Moreover, this technique was also used by Martucci et al. (2006) to correlate the creep response and the degree of cross-linking of gelatine-based films by glutaraldehyde. These authors indicated that gelatine-based bioplastics behave like a block copolymer build-up from triads of α-amino acids (glycine at every third position, soft blocks) and triads of hydroxyproline (proline and glycine as rigid blocks). The elastic modulus obtained for cross-linked gelatine was lower (25–80 MPa) than those obtained for HDPE and PVC (between 200 and 3500 MPa) (Xu et al. 2010).

Tensile Strength Tests

Tensile strength tests are used to obtain the stress–strain curve of a material. These tests consist of the continuous deformation of a material at a constant rate until breakdown. Most stress-strain curves of protein-based bioplastic materials are like those obtained from synthetic polymers being characterised by (1) an initial region where the material undergoes an elastic deformation at which the strain is proportional to the stress applied. The slope of this region is known as Young's modulus (E); (2) this first region is followed by a non-elastic deformation characterised by a decreased of the slope, reaching a maximum value ($\sigma_{\max}$); (3) eventually, the stress decreases as a consequence of the formation of a necking region, the cross-sectional area becomes smaller than the initial area, and the stress applied concentrates more in this reduced region, ending the experiment with the collapse of the probe ($\varepsilon_{\max}$) (Callister 2007). These tests have been applied to many biopolymers such as pea-, soy-, rice-, albumin-, and whey-based bioplastics (Felix et al. 2014, 2016b; Perez et al. 2016; Garavand et al. 2017; Gómez-Heincke et al. 2017). These measurements were useful to determinate changes in $\sigma_{\max}$ when the protein/plasticizer ratio changes (Felix et al. 2016b) or when some additives were introduced to the protein matrix (Felix et al. 2017; Cuadri et al. 2017). The tensile strength values depend highly on the processing technique and conditions used, as well as on the bioplastic composition. Verbeek and van den Berg (2010) summarised the main stress-strain parameters (E, $\sigma_{\max}$ and $\varepsilon_{\max}$) of protein-based bioplastics processed by extrusion, reporting that $\sigma_{\max}$ was between 2.9 and 25 MPa for soy-, zein-, and whey-based bioplastics. Moreover, E was between 18 and 2000 MPa, whereas $\varepsilon_{\max}$ was between 0.6% and 132%. On the other hand, several studies have reported these parameters for bioplastics processed by injection moulding, indicating that E ranged between 0.3 and 90 MPa, $\sigma_{\max}$ between 0.2 and 3 MPa, and $\varepsilon_{\max}$ between 0.6% and 15% for bioplastics based on crayfish, soy, albumen, rapeseed, and blood meal proteins under different processing conditions (Felix et al. 2014, 2017; Delgado et al. 2018; Álvarez-Castillo et al. 2019).

Moreover, the tensile-strength parameters for synthetic polymers typically range from 13 to 178 MPa for $\sigma_{\max}$, whereas the $\varepsilon_{\max}$ range between 500% and 80% for LDPE and polyesters, respectively (Guilbert et al. 2006). These results evidence that tensile parameters are typically higher for synthetic polymers.

5.2.3.2 Thermal Properties

The thermal properties of protein-based bioplastics are explained in a similar way to conventional polymers. When behaving like thermoplastic materials, these biopolymers can exhibit thermal events when subjected to thermal analysis (De Graaf 2000). Like in conventional polymers, the glass transition temperature (T_g), above which the mobility of biopolymer chains increases, is an essential parameter for material processing. In the case of biopolymers, this increase in protein mobility is a consequence of conformational changes, since physical interactions between biopolymer chains may change (Sochava and Smirnova 1993; Verbeek and van den Berg 2010). Hydrophobic and electrostatic interactions, as well as hydrogen bonding of amino acid residues, cause the native folding of proteins into quaternary, tertiary, and secondary structures. These interactions may undergo remarkable alterations when heating the sample that may lead to a shift from a glassy to a rubbery state. Moreover, this increase in protein mobility can be observed not only in final bioplastics but also in the protein/plasticizer blends obtained after mixing and before further processing (Irissin-Mangata et al. 2001). It should be borne in mind that this temperature depends on the amino acid sequence of the proteins, as well as on the plasticizer used (Chen et al. 2005). Hence, the processing of bioplastics should be performed above T_g, as the increase of protein chain mobility allows better chain rearrangements and proper mixing with plasticizer and additives (Felix et al. 2015a; Ricci et al. 2018).

Thermal properties can be assessed by several methods. Traditionally, there are three instrumental techniques that have been widely applied in polymer science: differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), and dynamic mechanical thermal analysis (DMTA), which can be applied under different deformation modes (bending, compression, tension, torsion, or shear).

DSC

When proteins are analysed by DSC, two different conformational changes can be typically determined: denaturation and glass transition. Both can appear in a thermogram; however, they can be differentiated since the former is irreversible and it is observed as a minimum in the curve, whereas the latter is a reversible second-order transition and is characterized by an inflection point. Apart from these thermal events, protein ageing is a reversible phenomenon that typically involves longer times than the experimental set-up (so, a return experiment is only observed during the first run). This thermal event is observed as a minimum in the curve, which typically appears at a lower temperature than protein denaturation (Ricci et al. 2018).

In order to differentiate these thermal events, two scans are typically performed when proteins are tested by DSC. Protein denaturation is observed in the first heating scan, but it is not observed in subsequent temperature ramps since it is irreversible. However, glass transition (responsible for T_g) is reversible, and it is observed in any heating or cooling temperature ramp. This analysis has been applied to protein powder, protein/plasticizer blends, and bioplastics (Felix et al. 2014). However, the most relevant information is obtained when it is performed to protein powder. This technique has been used to characterised soy protein isolate (Felix et al. 2014; Kweon et al. 2017), albumin protein isolate (Felix et al. 2014), egg yolk (Guerrero et al. 2004), wheat gluten (Jerez et al. 2005; Sun et al. 2007; Zarate-Ramirez et al. 2011), pea protein concentrate (Perez-Puyana et al. 2016), blood meal protein concentrate (Álvarez-Castillo et al. 2019), canola (Zhang et al. 2018), crayfish protein concentrate (Felix et al. 2015a), and whey protein isolated (Soukoulis et al. 2017), among others.

TGA

TGA measurements were performed to analyse the thermal stability of protein/plasticizer blends and protein-based bioplastics. Below 150 °C, the loss of volatile components and water leads to a decrease in sample weight (Jones et al. 2013; Ricci et al. 2018). In this sense, this technique was used to determine the hydrophilic character of proteins, since differences in moisture content after similar bioplastic processing have been related to the hydrophilic character of the proteins used in the formulation (Ricci et al. 2018). Loss of weight at higher temperatures have been associated with protein degradation (Jerez et al. 2007a). Although this interval depends on the protein system analysed, the temperature range typically is between 180 and 350 °C. Moreover, these thermograms also evidence the fat content of proteins isolated (Lukubira and Ogale 2013). Furthermore, this technique has also been used to determine the response of active ingredients such as calcium carbonate and citric acid aimed to produce porous bioplastics (Fernández-Espada et al. 2019). Sharma and Luzinov (2013) also studied whey-based binary bioplastics using this technique to determine the presence of volatile components after processing. This technique was also used for functionalized soy protein-based bioplastics with enhanced absorbent properties, to determine the thermal stability of samples (Cuadri et al. 2016). Furthermore, TGA was also used to analyse the thermal stability of sodium carbonate and bicarbonate when used as an additive in soy-based bioplastics obtained by injection moulding (Fernández-Espada et al. 2019).

DMTA

Although DMTA is clearly considered a rheological technique, it also provides useful information on thermal transitions for protein/plasticiser blends. This technique can measure the response of protein-based bioplastics, after the application of a small amplitude oscillatory test (using either bending, uniaxial tension or compression), as a function of temperature. A sinusoidal stress function is applied, and the sinusoidal strain response of the biopolymer material is measured (Panwar and Pal 2017). Results obtained from this technique are useful to understand the viscoelastic

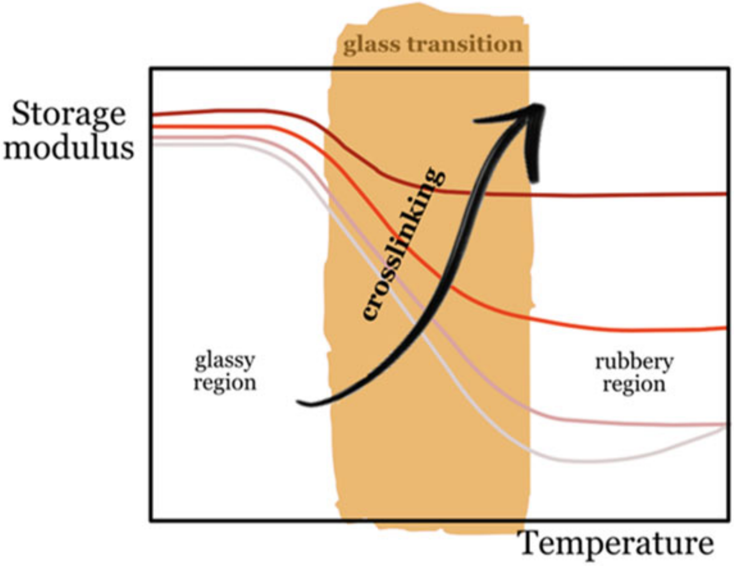

Fig. 5.4 Typical effect of protein cross-linking on the storage modulus. The lower curve shows a sometimes-found thermosetting potential of mild processed protein-based plastics

behaviour of biopolymers in relation to their molecular structure, being a complementary technique to the above-mentioned DSC and TGA analysis. From the oscillatory stress and strain functions, DMTA provides the storage modulus (E') and the loss modulus (E'') of viscoelastic materials during a thermal cycle (which may involve heating and/or cooling stages). Viscoelastic properties of protein-based materials change during heating or cooling, which allows the determination of the temperature for the glassy to rubbery transition (i.e., T_g) (Fig. 5.4).

DMTA technique has been used to characterised protein-based bioplastic materials and films, including a wide variety of proteins, such as albumen (Jerez et al. 2007b; Felix et al. 2017), soy (Felix et al. 2014), pea (Perez et al. 2016), wheat gluten (Jerez et al. 2005, 2007a; Zarate-Ramirez et al. 2011), rice (Felix et al. 2016b), blood meal (Verbeek and van den Berg 2011; Álvarez-Castillo et al. 2020b), and whey and canola (Shi and Dumont 2014a; Delgado et al. 2018). This technique has been key to determine changes in thermomechanical properties below and above service temperature. Moreover, this technique also has evidenced the thermosetting potential of bioplastics when the processing temperature of these materials was not enough to promote extensive protein cross-linking (Felix et al. 2015a).

5.2.3.3 Morphological Properties

Among the different microscopic techniques available, four are the techniques with major importance for the microstructural characterisation of bioplastics and films: confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM).

CLSM microscopy was often preferred to characterise the microstructure of protein-based films, exploiting the autofluorescence of proteins (Perotto et al. 2018). However, SEM is the most useful an extended technique for the characterisation of protein-based bioplastic microstructure, which comprises a wide variety of proteins: albumen (Felix et al. 2017), soy (Tummala et al. 2006; Felix et al. 2014; Fernández-Espada et al. 2016b), pea (Perez et al. 2016), wheat gluten (Jerez et al. 2005; Zárate-Ramirez et al. 2011, 2014b), rice (Felix et al. 2016b), and blood meal (Verbeek and van den Berg 2011; Álvarez-Castillo et al. 2020b), among others. SEM was also used to characterise the porous microstructure obtained after a dehydrothermal processing for bioplastics (Álvarez-Castillo et al. 2018). Moreover, this technique has also been used for the characterisation of the surface protein-based films and bioplastics (Oymaci and Altinkaya 2016; Thammahiwes et al. 2017). Furthermore, two approaches when using TEM microscopy has been made for the characterisation of protein-based bioplastic, considering that this technique requires electron transparency: (1) directly by thin bioplastic film (Sukyai et al. 2018) and (2) by cutting thin slides from bioplastic probes (Diañez et al. 2016; Felix et al. 2018). Eventually, surface topography has also been analysed through AFM microscopy, determining the nanometric three-dimensional structure of surfaces from protein-based bioplastics and films (Jerez et al. 2007b; Sukyai et al. 2018).

5.2.3.4 Optical Properties

Depending on the application selected for the protein-based bioplastic, some optical properties can be required (i.e., colour, reflection index, birefringence, transparency). Among them, transparency has been the focus of many researchers, especially for protein-based films. These optical properties are mainly influenced by two factors: protein system composition and processing conditions. The transmittance in the visible range (wavelength between 350 and 800 nm) has been measured in order to determine the transparency of the samples. For protein composition, Gounga et al. (2007) determined that transparency of WPI-based films increased when pullulan (microbial polysaccharide) content decreased. The protein composition refers not only to the inclusion of active ingredients but also to the nature of the protein used. For instance, Felix et al. (2014) found that albumen-based bioplastics are much more transparent than soy-based bioplastic, obtaining a high transparency level for bioplastics made with a mixture of albumen/soy proteins. Furthermore, Perez-Puyana et al. (2016) found that the transparency of pea-based bioplastics decreased when pea protein content increased. However, the transparency of protein-based bioplastics is also affected by the processing technique employed, as González-Gutiérrez et al. (2011) found that compression-moulding processing produced more transparent bioplastic materials than when they are processed by extrusion. Moreover, changes in bioplastic transparency can be observed even when these materials are manufactured using the same processing technique. Perez-Puyana et al. (2016) found that the transparency of pea-based bioplastic decreased with moulding time and increased with injection-moulding pressure.

5.2.3.5 Other Features Required for Protein-Based Bioplastics

Apart from the above-mentioned properties of bioplastics, other features can be required for these protein-based biodegradable materials. Some of these features required are related to the final use of the material, being in many cases aimed to be used in some specific application in specific fields (e.g. the food industry). The most relevant features to be considered for protein-based bioplastics and films are the following: gas permeation (barrier properties), antimicrobial activity, and superabsorbent capacity. Focused on packaging materials, gas permeation is required since it is key for preventing chemical and microbiological degradation (Di Pierro et al. 2005). In this sense, selective gas permeability is quite interesting for controlling gas exchanges during food storage (Razzaq et al. 2016), where the permeability of wheat gluten and gelatine-based films were tested, showing permeability to O_2, avoiding oxidation (Aguirre-Joya et al. 2018). Another issue that is considered relevant in the packaging industry is the development of active packages, where the implementation of antimicrobial activity has been of major interest, among the different applications proposed. Active packaging with antimicrobial properties is aimed to reduce, inhibit, or retard the growth of undesirable microorganisms. To achieve this feature two paths have been mainly considered: the addition of antimicrobial agents coating or adsorption of antimicrobials onto biopolymer surfaces (Martinez et al. 2013). In addition, the development of superabsorbent materials has been considered of great interest for hygienic and personal care applications. These materials display the ability to absorb and retain 10 times (1000%) their own weight of water, maintaining its integrity. Most superabsorbent materials are based on synthetic polymers from fossil fuels (e.g. from acrylic derivatives) which show poor biodegradability. The development of protein-based bioplastic matrices with superabsorbent capacity will involve a new and remarkable interest in these materials since it will contribute to reducing their toxicity, increasing their biodegradability, and reducing the dependence on the oil industry (Cuadri et al. 2017; Álvarez-Castillo et al. 2019).

5.2.4 Applications and Trends

5.2.4.1 Current Applications

The main parameters typically considered when bioplastics are characterised have been described in the previous section. The final application of the bioplastics developed will depend in turn on the accordance between service conditions required and the final properties of the materials. Mechanical properties of bioplastics like Young's modulus are typically required to be high in order to allow the final product to keep its shape when a proper consistency is required. However, excellent mechanical properties are not required in all cases. For instance, protein-based bioplastics exhibiting superabsorbent properties have been developed, displaying poorer mechanical properties (Fernandez-Espada 2016; Álvarez-Castillo et al. 2019). There are certain applications where high mechanical properties are not mandatory, such as matrices for nutrients release (Jiménez-Rosado et al. 2018). Nevertheless, these mechanical properties indeed can be required for some applications. This is the case of films and bioplastics with antimicrobial activity, although these materials have specific functionality, the mechanical properties should not diminish after the inclusion of the active ingredient (Seydim and Sarikus 2006; Campos et al. 2011; Martinez et al. 2013; Martín-Alfonso et al. 2014; Biscarat et al. 2015; Sharma and Singh 2016). Moreover, it must be highlighted the applicability of protein-based bioplastics in food packaging. In this case, food quality and safety during storage, extended shelf-life, reduced permeability to volatile compounds, oxygen, and moisture are pursued (Bradley et al. 2011). Thus, these protein-based bioplastics for packaging must be adequate to exert physical, physiochemical, and microbiological protection to maximise the quality-safety issues of the foods contained (Rhim et al. 2013; Dehnad et al. 2014). Eventually, there has been recently a high interest to produce bioplastics with enhanced superabsorbent properties (Cuadri et al. 2016), being this an added-value application with high potentials.

5.2.4.2 Future Trends

Processing technology of protein-based bioplastics has been extensively developed from the beginning of the twenty-first century, adapting processing methods from conventional polymers to the processing characteristics of proteins, where T_g and degradation temperatures delimit the temperature operational window for proteins. A reduction of mechanical properties has been observed too many times due to a high biodegradability or a plasticizer release (i.e., leaching). Thus, unaltered mechanical properties during their service live, together with proven environmental sustainability and adequate functionality, remain a challenge (Fig. 5.5). For example, a higher protein cross-linking during dough-like material processing or a post-curing stage after their processing could minimise limitations related to poor mechanical properties. Moreover, the production of bioplastics could be interlinked with biotechnology in order to maximise the use of food wastes, increasing the potential revenue of the entire bioprocessing chain. Although most processing techniques are

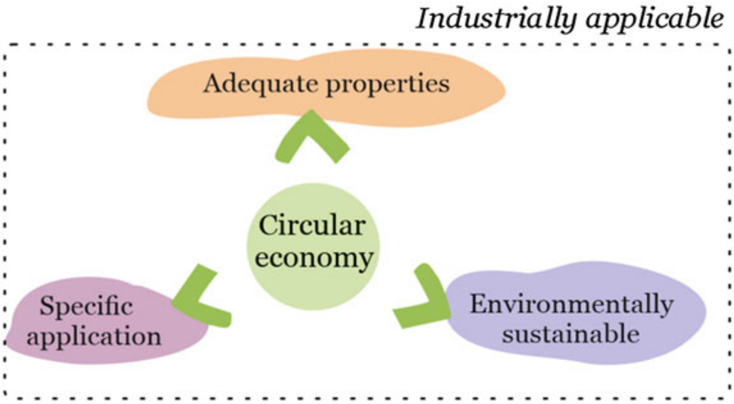

Fig. 5.5 Socio-economical requirements for future protein-based bioplastics

well-known and suitable materials have been developed, 3D printing still requires broad development for the manufacture of commercial products. In this sense, only fused deposition modelling has been used to process protein-based bioplastics. The analysis of other techniques which made use of other strategies (i.e., laser) can promote protein cross-linking, contributing to obtain materials with enhanced properties. The limitation related to the production scale of 3D printing can be neglected when mass production is not required. On the other hand, 3D printing opens the opportunity to increase process efficiency since it is the process that less energy requires per volume unit.

The increase in global population requires an increase in food production, at the same time as protein wastes and by-products are regarded to be used for animal feeding, instead of commodity productions. Moreover, climate change aims to increase forest area, which is to the detriment of using crops for bioplastic production. Consequently, it should be considered that bioplastic production could compete for arable land, fresh water and food production. The use of macro- and micro-algae by solar-driven photosynthetic processes brings important new routes to produce sustainable bioplastics, which would not be located on fertile land. Moreover, this route would capture CO_2 to produce protein-based bioplastic that can act as long-term carbon sinks.

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Conversion of Agro-industrial Wastes for the Manufacture of Bio-based Plastics

6

Larissa de Souza and Srividya Shivakumar

Abstract

Plastics have dominated nearly every avenue in business and industry since their discovery and mass manufacture in the 1900s. Their propensity to be moulded in numerous forms coupled with wide ranges of tensile strength has cemented their place in modern society. However, their durability has long become a cause for concern due to the staggering tonnes of plastic waste, generated annually, that persists in the environment, steadily damaging our planet. 'Bioplastics', specifically polyhydroxyalkanoates (PHA), present a viable solution to this problem. The terms 'biodegradable' and 'bio-based' are often used interchangeably with 'bioplastics' which isn't always accurate. This chapter begins with clear definitions of these important terms, in an effort to offer complete understanding, along with the current plastic waste scenario challenging our society. Furthermore, this chapter addresses the unexplored opportunities presented by the utilization of lignocellulosic agro-wastes for the industrial production of PHA (a costly prospect due to the high price of sugars). These substrates have the potential to greatly diminish the production costs, thereby ensuring an affordable end product. Lignocellulosic agro-wastes have escaped exploitation due to their protective lignin barrier. This chapter briefly summarizes certain pre-treatment techniques designed to overcome this hurdle through the removal of lignin, which is an important step in the efficient employment of lignocellulosic agro-wastes. Moreover, studies conducted in the past 5 years, dealing with the conversion of lignocellulosic agro-wastes (pre-treated and untreated) to PHA, have been discussed.

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Keywords

Lignocellulose · Pre-treatment · Delignification · Enzymatic saccharification · PHA production

6.1 Introduction

Plastics have dominated nearly every avenue in business and industry since their discovery and mass manufacture in the 1900s. Their versatility is attributed to their pocket-friendliness and propensity to be moulded in numerous forms coupled with a desirable tensile strength. However, the extreme durability of plastics has long become a cause for concern due to their persistence in the environment, generating staggering quantities of waste that, alarmingly, continues to increase per annum. Plastics are artificial, synthetic, high molecular weight polymers produced from the petro-chemical industry which can be moulded and set into a wide range of forms (Alauddin et al. 1995). The popular plastics in use, globally, are the thermoplastics: polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC) and polyethylene terephthalate (PET) (Kosior et al. 2006; Tokiwa et al. 2009). These petroleum- and oil-based commodities are non-biodegradable due to their high molecular weights, levels of crystallinity, degree of branching, intense melting temperatures (T_m), etc. (Kale et al. 2007; Tokiwa et al. 2009). The global production of plastics was at a steady increase at 359 million metric tonnes (Mt) in 2018 and included thermoplastics, polyurethanes, elastomers, adhesives and thermosets (Garside 2019). The highest consumer of plastics is the packaging industry with almost 35% from the global production. Analysis of the life cycle of plastics, from manufacture to recycling to waste from 1950 to 2015, revealed that the plastic waste generated was around 6300 million metric tonnes (Geyer et al. 2017). Waste plastics accumulate in the environment, undergoing physical deconstruction into microscopic pieces, which eventually find their way to every water body on the planet, especially oceans. Numerous studies in the past decade have shown that marine fauna ingest these tiny particles (Eriksen et al. 2014).

Globally, scientists have presented viable environmentally friendly alternatives that are collectively referred to as 'bioplastics'. However, this term requires further definition and fine-tuning as its meaning is often misunderstood, causing it to be used indiscriminately and inaccurately. Bioplastics can be defined in two modes: first, bioplastics could refer to the source of the raw materials used to manufacture the final plastic products (which doesn't necessarily mean that the product is biodegradable) and, second, bioplastics could refer to the biodegradability of the product in the environment through natural processes (Guillet et al. 1992; Tokiwa et al. 2009). Another commonly used term is 'bio-based' which refers to the origin of the raw materials utilized in the product. The term 'biodegradable' can be defined as a substance able to undergo decomposition into its constituents through the action of microorganisms and/or enzymes, without the production of toxic by-products (Guillet et al. 1992; Gautam 2009). These terms are often used as synonyms of each other, which, from their respective definitions, are not suitable. Over the years, governing authorities have set up standards, which include criteria and tests, to accurately determine the biodegradability of a material. Some examples include the European standard for packaging materials (EN13432), American Society for Testing and Materials (ASTM) standard D6400 for solid material biodegradation, International Organization for Standardization (ISO) standard 14855 for the determination of the ultimate aerobic biodegradability of plastic materials under controlled composting conditions, etc. (Kosior et al. 2006; Kale et al. 2007).

Polyhydroxyalkanoates (PHA) are polymers biosynthesized by microbes, triggered by the restriction of an essential nutrient and an abundant carbon source. These polymers function as spare resources to help the organism to endure stressful environmental situations. Producers of PHA span across multiple genera of Gram-positive and Gram-negative bacteria. Commercial PHA producers include *Azotobacter beijerinckii*, *Bacillus megaterium*, *Cupriavidus necator* (formerly *Ralstonia eutropha*), *Paracoccus denitrificans*, *Pseudomonas putida*, etc. PHA have innumerable qualities that render their high desirability as alternatives to popular non-biodegradable plastics. Molecular weights, ranging from 50,000 to 1,000,000 Da, are suitably high enough to produce PHA products, that are comparable to PP. Furthermore, PHA are completely biodegradable and biocompatible with elevated levels of polymerization and crystallization, insoluble in water, UV resistant, thermoplastic and non-toxic (Reddy et al. 2003; Bugnicourt et al. 2014). Unfortunately, the costly process of PHA production, due to the high expense of raw materials coupled with low efficiency, is responsible for the gradual deceleration of its manufacture, marketing and mass distribution (Bugnicourt et al. 2014; Wang et al. 2014).

Consequently, numerous studies over two decades have focussed on the conversion of wastes (from the food and agricultural sectors) to PHA, as potentially economic and renewable feedstocks. The most copious carbon source on the planet is lignocellulose (composed of varying concentrations of lignin and polysaccharides, i.e. cellulose and hemicellulose), which has escaped exploitation due to its recalcitrant barricade, lignin. Although lignin confers rigidity, strength, protection from pathogens and conduction of water and solutes, its inability to disintegrate severely hinders the utilization of the polysaccharides (Boerjan et al. 2003; Hassan et al. 2018), because of no accessibility of hydrolytic enzymes to the polysaccharides (Li et al. 2008). Thus, numerous pre-treatment procedures (Taherzadeh and Karimi 2008; Rabemanolontsoa and Saka 2016; Hassan et al. 2018) have been developed to remove this lignin hindrance, which is an important step in the efficient employment of lignocellulosic agro-wastes. Global lignocellulosic materials can be classified into one of four different categories, namely, grasses, agricultural biomass, softwoods and hardwoods. Agricultural biomass from cereal crops (straw, bagasse, etc.) is preferred as feedstocks due to their high content of cellulose, which can yield appreciable levels of fermentable sugars (Hassan et al. 2018) and can be directed for the production of PHA. This chapter briefly summarizes certain pre-treatment techniques and discusses the studies conducted in the past 5 years, dealing with the conversion of lignocellulosic agro-wastes (pre-treated and untreated) to PHA.

6.2 Pre-treatment of Lignocellulose

Lignocellulose comprises of varying levels of lignin, cellulose and hemicellulose. Lignin is a heteropolymer that is composed of monolignols with numerous types of bonds, the majority of which are β-aryl ether (β-O-4) linkages (Wong 2009). Part of the polysaccharide component, cellulose, is built from D-glucose units joined together with β-1,4-glycosidic bonds. This polymer has a crystalline structure and is insoluble in water and indigestible to humans. The other polysaccharide component, hemicellulose, is built from multiple pentose sugars like xylose, arabinose, galactose, mannose, etc. Xylose is usually found in higher quantities than the other pentoses (Wong 2009; Jiang et al. 2016).

Pre-treatment is a crucial step required to eliminate lignin from lignocellulose. The term 'pre-treatment' is defined as the removal of lignin from lignocellulose for accessibility to cellulose and hemicellulose. A commonly used synonym, for this process, is delignification. Pre-treatment increases the porosity of the biomass, after which its polysaccharide component can be efficiently processed. Additionally, pre-treatment diminishes the crystallinity of cellulose and contributes to the material's processability. The degree of polymerization of cellulose is in the range of 800–10,000, preventing effective cellulosic conversion to sugars, which is overcome via pre-treatment through reduction of polymerization levels. Furthermore, many pre-treatment techniques target the hemicellulose component (which bonded to both cellulose and lignin), thereby enhancing cellulose hydrolysis and digestibility (Sawant et al. 2016; Kim 2018).

Efficient pre-treatment techniques are distinguished based on certain criteria which include simplicity, being inexpensive, being environmentally safe, producing desirable products (like fermentable sugars, chemical precursors, etc.), zero production of toxic derivatives, decreased crystallization of the polysaccharide layers, decreased sugar losses, low energy inputs, etc. The pre-treatment processes for lignocellulose (Fig. 6.1) can be broadly classified into four main groups, namely, (1) physical, (2) chemical, (3) physico-chemical and (4) biological (Hassan et al. 2018). However, in light of recent advancements in the field of genetic engineering, the authors propose a fifth group, namely, (5) genetic adjustment. At present, a singular pre-treatment method to tackle every type of lignocellulosic biomass does not exist (Hassan et al. 2018; Kim 2018).

An accompanying step, to the pre-treatment process of lignocellulosics, is the utilization of commercial or crude hydrolytic enzymes (mostly cellulases) to act on the pre-treated biomass for the release of fermentable sugars. This routine step is called enzyme saccharification (Kumar and Sharma 2017) and ensures efficient exploitation of pre-treated lignocellulose. Efficient saccharification is impossible in the absence of delignification, the reason being that the intact lignin barrier blocks the contact of hydrolytic enzymes with the polysaccharide layers of lignocellulose (Li et al. 2008). The following subsections, of this chapter, briefly summarize each category of pre-treatment and discuss the progress of the conversion of lignocellulosic agro-wastes to PHA achieved in the last 5 years (Table 6.1).

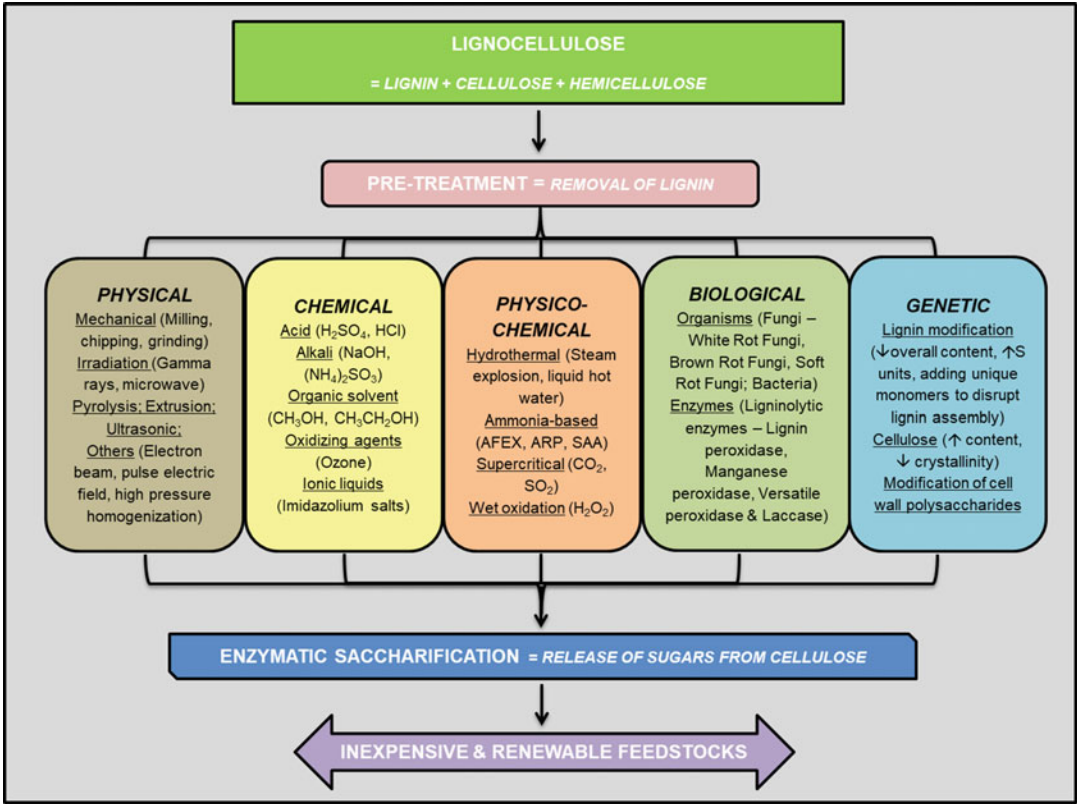

Fig. 6.1 Representation of the processing of lignocellulose towards inexpensive and renewable feedstocks; *AFEX* ammonia fibre explosion, *ARP* ammonia recycle percolation, *SAA* soaking aqueous ammonia, *S* syringyl units found in lignin

Table 6.1 Summary of research conducted for the conversion of lignocellulosic agro-industrial wastes to PHA

Lignocellulosic biomass	Type of pre-treatment	Enzyme saccharification	PHA producer	Type of PHA	Yield	References
Ensiled grass press juice (EGPJ)	Physical	N.S.	*Burkholderia sacchari* IPT101	PHB	33%	Cerrone et al. (2015)
Ensiled grass press juice (EGPJ)	Physical	N.S.	*Pseudomonas chlororaphis* IMD555	mcl PHA	10%	Cerrone et al. (2015)
Chicory roots	N.S.	Viscozyme®	*Cupriavidus necator* DSM 428	PHB	66%	Haas et al. (2015)
Chicory roots	N.S.	Viscozyme®	*Cupriavidus necator* DSM 531	PHB	46%	Haas et al. (2015)
Chicory roots	N.S.	Viscozyme®	*Cupriavidus necator* DSM 545	PHB	78%	Haas et al. (2015)
Spruce wood sawdust	Physico-chemical	N.S.	*Cupriavidus necator*	PHBV	60.5 g/L	Koller et al. (2015)
Lignin	None	None	*Oceanimonas doudoroffii*	N.S.	N.S.	Numata and Morisaki (2015)
Rice bran	N.S.	N.S.	*Ralstonia eutropha* NCIMB 11599	PHB	90.1 wt %	Oh et al. (2015)
Rice bran	N.S.	N.S.	Recombinant *E. coli* XL1-Blue	PHB and PHBL	97.2 wt %	Oh et al. (2015)
Rice bran	N.S.	N.S.	Recombinant *R. eutropha* 437-540	PHBL	N.S.	Oh et al. (2015)
Rice straw	Chemical	N.S.	*Ralstonia eutropha* ATCC 17699	PHB	11.42	Saratale and Oh (2015)
Rice straw	Chemical	N.S.	*Bacillus cereus* PS 10	PHB	10.61	Sharma and Bajaj (2015)

Corn stover		Cellulase from *Trichoderma reesei* and *Aspergillus niger*	*Paracoccus* spp. LL1	N.S.	72.4%	Sawant et al. (2015)
Pineapple pieces and sugarcane bagasse	None	None	*Bacillus* spp.	PHB	1.86 g/L	Suwannasing et al. (2015)
Rice straw	Physico-chemical	N.S.	*Cupriavidus necator*	PHBV	N.S.	Ahn et al. (2016a, b)
Wheat bran	Chemical	Cellulase from *Trichoderma reesei* and β-glucosidase from *Aspergillus niger*	Mutant *Ralstonia eutropha* NCIMB 11599	N.S.	62%	Annamalai and Sivakumar (2016)
Sunflower stalks	Hydrothermal	Cellic CTec3	Recombinant *Ralstonia eutropha* NCIMB 11599	PHB	7.86	Kim et al. (2016)
Sugarcane bagasse	Biological	N.S.	*Pandoraea* spp. ISTKB	PHBV	N.S.	Kumar et al. (2016)
Rubber seed oil	None	None	*Bacillus cereus*	PHB	2.16 g/L	Kynadi and Suchithra (2017)
Sugarcane trash	Chemical pre-treatment with acid	N.S.	*Bacillus* spp.	PHBV	73%	Moorkoth and Nampoothiri (2016)
Pineapple peels	None	None	*Ralstonia eutropha*	N.S.	N.S.	Vega-Castro et al. (2016)
Corn stover	Chemical	N.S.	*Pseudomonas putida* KT2440	N.S.	1 g/L	Liu et al. (2017)

(continued)

Table 6.1 (continued)

Lignocellulosic biomass	Type of pre-treatment	Enzyme saccharification	PHA producer	Type of PHA	Yield	References
Sugarcane bagasse (SCB)	Chemical	N.S.	*Halogeometricum borinquense* E3	PHBV	N.S.	Salgaonkar and Bragança (2017)
Plant and algae biomass	None	None	*Co-culture of Saccharophagus degradans* 2-40 and *Bacillus cereus*	N.S.	34.5%	Sawant et al. (2017)
Kraft lignin (KL)	Chemical	N.S.	*Cupriavidus basilensis* B-8	Polymer with monomers: (S)-3-hydroxybutanoic acid (S3HB), (R)-3-hydroxybutyrate (R-3HB) and 3-hydroxybutanoic acid (3HB)	319 mg/L	Shi et al. (2017)
Rice straw	Biological	N.S.	*Cupriavidus basilensis* B-8	N.S.	N.S.	Yan et al. (2017)
Sugarcane	None	None	Co-culture of *Ralstonia eutropha* 5119 and *Bacillus subtilis*	PHBV	66%	Bhatia et al. (2018)
Rice husks	Chemical	Celluclast 1.5 L and Novozyme 188	*Burkholderia cepacia* USM (JCM 15050)	N.S.	7.8 g/L	Heng et al. (2017)
Waste office paper (WOP)	Chemical	N.S.	*Cupriavidus necator*	N.S.	4.45 g/L	Neelamegam et al. (2018)
Date seed oil and date molasses	None	None	*Cupriavidus necator* H16 Re2058/pCB113	PHBH	80%	Purama et al. (2018)
Red seaweed *Gelidium amansii*	None	None	*Saccharophagus degradans* 2-40	N.S.	27%	Sawant et al. (2018)

Rice straw	Physico-chemical	Innate hydrolytic enzymes from PHA producer	*Cupriavidus basilensis* B-8	N.S.	0.48 g/L	Si et al. (2018)
Barley biomass hydrolysate (BBH)	Chemical	N.S.	*Ralstonia eutropha*	N.S.	1.8 g/L	Bhatia et al. (2019)
Miscanthus biomass hydrolysate (MBH)	Chemical	N.S.	*Ralstonia eutropha*	N.S.	2.0 g/L	Bhatia et al. (2019)
Pine biomass hydrolysate (PBH)	Chemical	N.S.	*Ralstonia eutropha*	N.S.	1.7 g/L	Bhatia et al. (2019)
Green macroalgae	N.S.	N.S.	*Haloferax mediterranei*	PHBV	2.2 g/L	Ghosh et al. (2019)
Tequila agave bagasse (TAB)	Chemical	N.S.	*Burkholderia sacchari*	PHB	24 g/L	González-García et al. (2019)
Alkaline pre-treatment liquor (APL)	Chemical	N.S.	*Cupriavidus necator* DSM 545	N.S.	2.1 g/L	Li et al. (2019)
Cassava	N.S.	N.S.	*Halogeometricum boringuense* E3	PHBV	1.52 g/L	Salgaonkar et al. (2019)
Kenaf biomass	N.S.	N.S.	*Ralstonia eutropha*	PHB	10.10 g/L	Saratale et al. (2019)
Wheat straw hydrolysate	N.S.	N.S.	*Ralstonia eutropha*	PHB	12.1 g/L	Soto et al. (2019)
Lignin	None	None	*Pseudomonas putida* AG2162	mcl PHA	N.S.	Salvachúa et al. (2020)

(continued)

Table 6.1 (continued)

Lignocellulosic biomass	Type of pre-treatment	Enzyme saccharification	PHA producer	Type of PHA	Yield	References
Ferulic acid	None	None	*Pseudomonas putida* KTc9n20	N.S.	270 mg/L	Zhou et al. (2020)

The research represented above span from the last 5 years

None = direct conversion of lignocellulosic agro-wastes to PHA

N.S. not specified, *PHB* poly(3-hydroxybutyrate), *PHBV* copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate), *PHBH* copolymer poly (3-hydroxybutyrate-co-3-hydroxyhexanoate), *PHBL* copolymer poly(3-hydroxybutyrate-co-lactate), *mcl PHA* medium-chain-length PHA

6.2.1 Physical Pre-treatment

The physical pre-treatment of lignocellulosics aim to disrupt the structural integrity of lignin through reduction of the particle size, thereby increasing enzymatic accessibility to the underlying polysaccharide layers. Additionally, physical pre-treatments achieve reduction in polymerization and crystallinity levels of the substrates (Mood et al. 2013; Sun et al. 2016). The disadvantage of this pre-treatment is that this technique requires specialized equipment, high energy inputs, intense temperatures (sometimes accompanied by elevated pressure), etc., which contribute to the costliness of pre-treatment (Mood et al. 2013; Kumar and Sharma 2017).

6.2.1.1 Types

There are numerous subcategories of physical pre-treatment (Fig. 6.1) like mechanical (grinding, milling or chipping), irradiation (microwave, gamma rays), pulse electric field, pyrolysis, extrusion, etc., which have been extensively reviewed (Taherzadeh and Karimi 2008; Mood et al. 2013; Sun et al. 2016; Kumar and Sharma 2017) but will not be discussed.

6.2.1.2 Conversion of Physically Pre-treated Agro-wastes to PHA

Minimal studies have been performed on the conversion of physically pre-treated agro-wastes to PHA. Much of agricultural waste is used as fodder for livestock in the form of ensilage. This is the fermented fodder obtained after compressing and storing substrates like cornstalks, oats, rye, millet, etc., while still fresh into silos (pits or vats). A study was performed on the usage of ensiled grass press juice (EGPJ) as the singular source of carbon for the production of PHA by *Burkholderia sacchari* IPT101 and *Pseudomonas chlororaphis* IMD555. The former strain accumulated 33% poly(3-hydroxybutyrate) (PHB), while from the latter, 10% medium-chain-length PHA was obtained (Cerrone et al. 2015).

Physical pre-treatments are routinely used in combination with other pre-treatment processes. A study was conducted using sunflower stalks, which were hydrothermally pre-treated at 190 °C for 5 min with stirring at 800 rpm. The slurry was rapidly cooled and centrifuged to obtain the pre-treated biomass. The processed biomass was then subjected to enzymatic saccharification with Cellic CTec3, to obtain the hydrolysate to be used for PHA production. The PHA producer used in this study was recombinant *Ralstonia eutropha* NCIMB 11599, engineered to express xylose-utilizing *E. coli xylAB* genes, for PHA biosynthesis. The production of PHA from the hydrolysate of pre-treated sunflower stalk, containing 16.8 g/L of glucose and 5.9 g/L of xylose, yielded 7.86 g/L PHB with content of 72.53 wt% (Kim et al. 2016). In another study, rice straw was subjected to acid hydrolysis with varying concentrations of sulphuric acid in order to generate hydrolysates with desirable levels of levulinic acid, for the production of copolymer poly (3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV) or PHBV]. However, acid hydrolysis alone was unable to yield the required levels, and thus, heat pre-treatment of the acid hydrolysate was carried out. PHA production from the hydrolysate, of 2% sulphuric acid digestion and heat treated for 60 min, yielded a copolymer with 22.9% 3HV. Additional heating decreased productivity, perhaps due to the inhibitory effects of intermediates (elevated quantities) in the hydrolysate (Ahn et al. 2016a). Furthermore, the research was refined to enhance the copolymer production by adjusting the concentration of the hydrolysate of acid pre-treated rice straw with the production medium. *Cupriavidus necator* yielded suitable quantities of [P(3HB-co-3HV)] with hydrolysate-to-media (H/M) ratio of 2:8. Moreover, the technique of introducing the fermentation medium was investigated and revealed that the repeatedly fed method was the best for generating a copolymer with 20–30% 3HV, suitable for application in the medical field (Ahn et al. 2016b).

6.2.2 Chemical and Physico-chemical Pre-treatment

Generally, chemical pre-treatments involve the usage of acid and alkali for pre-treatment. Acid pre-treatment is used to solubilize hemicellulose and increase the accessibility of hydrolytic enzymes to cellulose. Alkali pre-treatment functions to remove lignin through the cleavage of ester bonds and decreases the crystallinity of cellulose. Although chemical pre-treatment is advantageous due to the short reaction time, the major disadvantage is the production of toxic by-products (like formic acid, vanillin, furfural, hydroxymethylfurfural, etc.) formed in the pre-treatment processing. These inhibitory products require extra steps for removal before directing the hydrolysates for usage as raw materials. Additionally, the equipment corrosion, problems associated with the recovery of chemicals, etc. add to the shortcomings of this technique (Mood et al. 2013; Yang et al. 2014; Sun et al. 2016).

Physico-chemical pre-treatments involve the combination of certain chemical pre-treatments with physical ones in an attempt to enhance the pre-treatment process by reducing the reaction time, lowering the production of toxic by-products, increasing the conversion efficiency, etc. (Yang et al. 2014; Rabemanolontsoa and Saka 2016). The most popular technique is steam explosion and achieves ‘explosive deconstruction’ of lignocellulose. This is caused by a sudden and abrupt change in surrounding pressure, resulting in physical degradation of the recalcitrant structures (Kim 2018). This technique is thought to be relatively eco-friendly and energy-friendly (Yang et al. 2014). A related technique, called liquid hot water, uses water at different temperatures to solubilize lignin and hemicellulose component (which is present as oligomers in the solution, thus avoiding the production of undesirable by-products).

6.2.2.1 Types of Chemical and Physico-chemical Pre-treatments

There are numerous subcategories of chemical and physico-chemical pre-treatments (Fig. 6.1). For example, dilute acid, concentrated acid, ozonolysis, organic solvents, ionic liquids, etc. belong to chemical pre-treatments, and ammonia fibre expansion (AFEX), ammonia recycle percolation (ARP), and wet oxidation (hydrogen peroxide) are included in physico-chemical pre-treatments. Comprehensive details of each technique are available in literature (Taherzadeh and Karimi 2008; Mood et al. 2013; Sun et al. 2016; Kumar and Sharma 2017).

6.2.2.2 Conversion of Chemically Pre-treated Agro-wastes to PHA

Innumerable studies have been conducted on rice straw as a potentially inexpensive and renewable raw material for the production of PHA. Most studies have used chemical pre-treatment techniques to make the substrate amenable to processing and enzymatic hydrolysis. In one study, rice straw was chemically pre-treated with sulphuric acid in a two-step process which involved pre-treatment with 1% acid at 121 °C for 45 min and then pre-treatment with 5% acid at 121 °C for 60 min, taken in the solid/liquid ratio, 1:10. The hydrolysates were tested for PHA production by *Bacillus cereus* PS 10, which yielded 10.61 g/L PHA. The organism showed preference for the hydrolysate medium over glucose-based medium, producing 8.6 g/L PHA on the latter. Response surface methodology (RSM) for optimization resulted in an enhanced yield of 23% PHA accumulation. The polymer produced was found to be PHB (Sharma and Bajaj 2015). In another study, rice straw was subjected to pre-treatment with alkali (2% NaOH, 121 °C, 30 min) and enzyme hydrolysis, which yielded 703 mg/g sugars. These were directed for PHA production by *Ralstonia eutropha* ATCC 17699 that supplied 11.42 g/L PHA with 75.45% accumulation under optimized conditions at 48 h. As in the previous study, the polymer was determined as PHB (Saratale and Oh 2015). Rice husks were chemically pre-treated using potassium hydroxide (KOH) at elevated temperature and pressure levels (121 °C, 0.1 MPa) and then subjected to enzyme saccharification with Celluclast 1.5 L (EC 3.2.1.4) and Novozyme 188 (EC 3.2.1.21). The resultant sugar yield was up to 87% of the total carbohydrate content. The sugars were utilized by *Burkholderia cepacia* USM (JCM 15050) and an engineered strain of *Cupriavidus necator* H16, *Cupriavidus necator* NSDG-GG, for PHA production. *B. cepacia* USM generated 7.8 g/L PHA and utilized the hydrolysates much effectively than *C. necator* NSDG-GG (Heng et al. 2017).

Another popular agro-waste is wheat bran and has been studied extensively. Recently, wheat bran was pre-treated, chemically, with alkali and then hydrolysed with cellulase from *Trichoderma reesei* (37 FPU/g) and β-glucosidase from *Aspergillus niger* (50 CBU/g). The sugars released were found to be glucose and xylose. A mutant strain, *Ralstonia eutropha* NCIMB 11599, was used for PHA production utilizing the wheat bran hydrolysates. The resultant PHA accumulation was 62.5% with productivity of 0.255 g/L/h (Annamalai and Sivakumar 2016). In another study, alkaline pre-treatment liquor (APL) was subjected to optimization through the Plackett-Burman (PB) and central composite designs (CCD) for its conversion to PHA, using *Cupriavidus necator* DSM 545. The experiments were based on the supplementation of different chemicals, namely, oxidative enzymes (laccase, aryl-alcohol oxidase), mediators (ABTS, HOBT), DMSO, silica nanoparticle Aerosol R816 and surfactant Tween 80. Through optimization, the supplementation of R816, ABTS and Tween 80 achieved a tenfold increase in the PHA yield, 2.1 g/L (Li et al. 2019).

Studies have been conducted on corn-based agro-wastes in the past decade. Recently, corn stover was chemically pre-treated with acid (1% sulphuric acid for 30 min) and alkali (1% sodium hydroxide for 60 min) at 120 °C. This generated an increase of 11.2% and 8.3% for glucan and xylan, respectively. In the same study, *Pseudomonas putida* KT2440 was subjected to PHA production from lignin, resulting in 1 g/L PHA (Liu et al. 2017).

In recent times, corporate waste has grabbed attention as a potential substrate for PHA production. One such study evaluated the potential of PHA production from waste office paper (WOP). The substrate was chemically pre-treated with acid (0.5% sulphuric acid at 121 °C for 30 min) and then subjected to hydrolysis with enzyme. The WOP hydrolysate obtained (containing mostly glucose and xylose) was used as the feedstock for the production of PHA by *Cupriavidus necator*. The PHA production was found to be 4.45 g/L with accumulation of 57.52% (Neelamegam et al. 2018), which is comparable to numerous PHA producers studied. Another interesting substrate studied was bagasse from the tequila industry, tequila agave bagasse (TAB). The substrate was chemically pre-treated yielding 20.6 g/L sugars with the production of inhibitory phenolic compounds. The hydrolysate was detoxified with activated charcoal, showing the maximum percentage of removal (92%), and this detoxified hydrolysate was used for PHA production (two-step batch fermentation) by *Burkholderia sacchari*, an organism capable of consuming xylose. The PHA yield was found to be 24 g/L, and the polymer was PHB (González-García et al. 2019).

Commercial producer, *Cupriavidus necator*, has been investigated for PHA production using inexpensive sources and has been successful at small scale. In a study on the utilization of softwood for PHA production, spruce wood sawdust was subjected to microwave pre-treatment (300 and 500 W) in the presence of glycerol and *p*-toluenesulfonic acid, resulting in a dark viscous liquid. After filtration to remove residual particulates and neutralization, the liquid was used as the raw material for PHA production. The hydrolysate served as an inexpensive precursor for 3-hydroxyvalerate (3HV). The PHA producer, *Cupriavidus necator*, in the bioreactor fermentation yielded 60.5 g/L PHBV with PHA accumulation of 77%. The concentration of 3HV in the polymer was found to be 0.8% and was enough to produce the desired mechanical properties (Koller et al. 2015). In a study with a different species of *Cupriavidus*, kraft lignin (KL) was studied for conversion to PHA. KL is the by-product of the kraft process, which is a technique that converts wood chips to pulp through removal of lignin. This is the main process used in the pulp and paper industry. KL contains many toxic by-products and is difficult to dispose. *Cupriavidus basilensis* B-8 was grown on medium containing KL (5 g/L concentration) as the singular source of carbon and was found to produce 128 mg/L PHA. Through fed batch fermentation, the yield was enhanced to 319.4 mg/L. Surprisingly, the polymer was found to contain 98.3 mol% of (S)-3-hydroxybutanoic acid (S3HB), 1.3 mol% of (R)-3-hydroxybutyrate (R-3HB) and 0.4 mol% of 3-hydroxybutanoic acid (3HB) (Shi et al. 2017).

Halophiles are organisms that require growth environments that are high in salt content (10–20%). They can be found in all three domains (Kourmentza et al. 2017). However, the Haloarchaea have been acquiring attention over the last decade as producers of PHA, from numerous inexpensive raw materials. A study with *Halogeometricum borinquense* strain E3 for PHA production from the hydrolysates of dilute acid (0.75% (v/v) sulphuric acid) pre-treated sugarcane bagasse (SCB) was conducted. The PHA productivity by the organism, on sodium chloride (NaCl) medium supplemented with varying concentrations of SCB hydrolysate (25% and 50%), was 3.0 (mg/g/h) and 2.7 (mg/g/h), respectively. Additionally, polymer analyses elucidated that the copolymer P(3HB-co-3HV) was produced with 13.29 mol% 3HV (Salgaonkar and Bragança 2017). Furthermore, the organism was evaluated for its potential to produce PHA from cassava waste (CW) and starch. The PHA yields obtained were 1.52 g/L and 4.6 g/L, respectively. Characterization of the polymer confirmed it to be P(3HB-co-3HV) which comprised of 19.65% and 13.11% 3HV units, respectively (Salgaonkar et al. 2019). A separate study was conducted where *Haloferax mediterranei* was cultured on a special medium made from the hydrolysates of green macroalgae, *Ulva* sp. (25% v/v), for the production of PHA and achieved 2.2 g/L PHA. The polymer was found to be P(3HB-co-3HV) (Ghosh et al. 2019). These halophilic organisms have great potential as commercial PHA producers. They require seawater over freshwater which can be applied in unsterile conditions due to the elevated salt contents that are inhibitory to most contaminants, and thus, this contributes to low energy inputs. Additionally, the polymer extraction process is greatly simplified with the use of isotonic water that will induce hypo-osmotic shock in the halophiles, thereby rupturing the cell and releasing the polymer granules (Kourmentza et al. 2017).

6.2.3 Biological Pre-treatment

Biological pre-treatment is acquiring attention due to its eco-friendly approach involving the elimination of lignin through the action of white rot fungi (WRF), nature's lignin degraders. WRF, belonging to *Basidiomycota*, produce potent oxidative enzymes, called ligninolytic enzymes (LE), which are responsible for lignin degradation. These include lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16) and laccase (EC 1.10.3.2) and are assisted by accessory enzymes. WRF attack lignin, solubilizing it through the formation of water-soluble fragments, and further mineralize lignin via the formation of CO_2 (Blanchette 1995; Wong 2009). Biological pre-treatment has countless advantages over other pre-treatment processes. This technique executes pre-treatment through solid-state fermentation (SSF) where the organism comes in direct contact with the substrate, in low water conditions. SSF is preferred as it mimics the natural habitat of the WRF and, thus, limits the time needed for organisms' acclimatization. The other factors contributing to the environmentally safe approach of biological pre-treatment are the absence of toxic derivatives (like furfural, hydroxymethylfurfural, vanillin, etc.); lack of high energy, temperature and pressurized conditions; lack of expensive equipment; etc., which are routinely used in most physical, chemical and physico-chemical processes. However, the one disadvantage of biological pre-treatment is the prolonged time period of the process (Malherbe and Cloete 2002; Thomas et al. 2013; Rabemanolontsoa and Saka 2016).

6.2.3.1 Types of Biological Pre-treatment

Biological pre-treatment can be accomplished in two ways: through the employment of (1) WRF and (2) ligninolytic enzymes (LE) harvested from WRF. WRF can be classified into two factions: type 1 organisms have selective degradation affinity for lignin with negligible hydrolysis of cellulose/hemicellulose and type 2 organisms have collective degradative affinity for lignocellulose as a whole (Wong 2009). Examples of type 1 organisms are *Ceriporiopsis subvermispora*, *Pleurotus ostreatus*, *Phlebia radiata*, etc., and type 2 organisms include *Phanerochaete chrysosporium*, *Trametes versicolor*, *Irpex lacteus*, etc. Generally, type 1 organisms are preferred as they enrich the biomass with cellulose through their selective degradation of lignin. In addition, some WRF produce hydrolytic enzymes (Wong 2009; Dashtban et al. 2010) which achieve saccharification of the underlying polysaccharide layers, and thus, these organisms can perform the dual role of bio-delignification and bio-saccharification.

Ligninolytic enzymes comprise of haem peroxidases and phenol oxidases. The peroxidase system includes lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). These enzymes require hydrogen peroxide (produced by the cell or externally present) as a co-substrate to stimulate their activity. LiP is substrate non-specific and catalyses multistep electron transfer reactions of phenolic compounds via formation of intermediate radicals that undergo side-chain cleavage, demethylation, etc. Unlike other peroxidases, LiP can also oxidize non-phenolic lignin compounds with β-1 and β-O-4 linkages, without mediators. MnP oxidizes Mn(II) to Mn(III) and forms a complex with chelators (like oxalate). This complex is a diffusible redox mediator that reacts with phenolic units in the lignin structure. MnP finds great use in the decolourization of industrial dyes and effluents. VP, a relatively recent discovery, is a hybrid enzyme with the dual oxidative ability to catalyse the oxidation of Mn(II) and aromatic (phenolic and non-phenolic) units (Wong 2009; Dashtban et al. 2010). The phenol oxidases include laccase (LAC) and uses molecular oxygen as its electron acceptor. Laccases are blue multi-copper oxidoreductases that catalyse the oxidation of aromatic and non-aromatic units in lignin through the formation of free reactive radicals, bringing about depolymerization reactions (Dashtban et al. 2010).

6.2.3.2 Conversion of Biologically Pre-treated Agro-wastes to PHA

Numerous studies on the conversion of agro-wastes to PHA have been undertaken with a focus on either chemical pre-treatments or on the direct action of the PHA producer on the substrate, without any pre-treatment. In the last 5 years, minimal studies have explored the use of biological pre-treatment of lignocellulose with WRF and/or ligninolytic enzymes, for the ultimate goal, conversion of lignocellulose to PHA. In certain studies, enzyme saccharification was performed without prior pre-treatment. Enzymatic hydrolysis of corn stover was carried out using a cellulase cocktail prepared from the co-culture of *Trichoderma reesei* and *Aspergillus niger*. The sugars liberated through this process were directed for PHA production using *Paracoccus* spp. LL1 and yielded PHA accumulation of 72.4% (Sawant et al. 2015).

A viable strategy for ensuring optimum sugar levels from biologically pre-treated biomass is to choose a lignin-degrading organism, which exhibits preference for lignin over cellulose. Further, the lignin degrader should either have little or no cellulase-producing capacity. Such a strategy was adopted in a study conducted to isolate and study bacterial species capable of lignin biodegradation. *Pandoraea* spp. ISTKB, a Gram-negative, non-spore-producing, motile bacterium, was isolated and found to produce lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (LAC). Additionally, it produced hydrolytic enzyme, xylanase, but not cellulases (Kumar et al. 2015, 2016). This organism demonstrated higher delignification on sugarcane bagasse in submerged fermentation conditions with lignin and hemicellulose removal at 10% and 20%, respectively. Pre-treatment with this organism resulted in a 3.7-fold increase in the enzymatic saccharification, post delignification (Kumar et al. 2016). Furthermore, this organism was investigated for its potential to produce PHA from lignin and its derivatives (like vanillic acid, 4-hydroxybenzoic acid and kraft lignin). The highest PHA yield was obtained from 4-hydroxybenzoic acid, and the polymer extracted was elucidated as the copolymer, PHBV. Through optimization using response surface methodology, the PHA production was improved by 66% (Kumar et al. 2017). This is one of the few promising studies conducted where a bacterium is used for biological pre-treatment and, further, for PHA production.

In a recent study, a novel strategy for bioconversion of lignocellulose to PHA was investigated. Rice straw was pre-treated with dilute acid and then subjected to further processing by *Cupriavidus basilensis* B-8 that could act on lignin droplets formed in dilute acid pre-treatment (DAP). In this pre-treatment strategy, bacteria-enhanced dilute acid pre-treatment (BE-DAP) increased the enzymatic digestibility of the substrate by 35–70% in chemically pre-treated biomass and by 173–244% in untreated substrate (Yan et al. 2017). This strategy targeted the pre-treatment process, DAP, using a bacterium to further degrade the acid pre-treated rice straw, thus exposing the underlying polysaccharides to enzyme hydrolysis.

6.2.4 Genetic Adjustment

Genetic adjustment of lignocellulose targets the lignin biosynthesis pathway (specifically, biosynthesis of lignin monomers) of model species of certain softwoods (gymnosperms) and hardwoods (angiosperms). The lignin biosynthesis cycle has undergone revision in the past two decades with the addition of new information obtained through genetic studies. Briefly, lignin biosynthesis begins with the formation of the monolignols (derivatives of cinnamic acid) through the phenylpropanoid pathway. The main monolignols are *p*-coumaryl, coniferyl and sinapyl alcohols. Next, the newly synthesized monolignols are transported to the cell wall through the action of plant peroxidases and laccases. And finally, the monolignols undergo oxidization and lignification, giving rise to *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) phenylpropanoid units, respectively (Boerjan et al. 2003; Vanholme et al. 2010; Liu et al. 2018). The process through which monolignols (usually at the β-position) are joined to the growing chain of the heteropolymer via 'end-wise' reactions is called lignification (Boerjan et al. 2003). Genetic adjustment of lignocellulosic flora is gaining momentum as a potential pre-treatment technique because of its capacity to engineer customized vegetation, to suit the requirements of different industries. For example, the pulp and paper industries spend immensely for pre-treatments to remove lignin, and concentrate the cellulose fibres, from wood. Genetic manipulation can alter these plant species to produce, say, less lignin and more cellulose, thereby potentially reducing the costs associated with pre-treatment.

6.2.4.1 Strategies for Genetic Adjustment of Lignin

Genetic studies, investigating the monolignol biosynthetic pathway, have elucidated certain strategies that have potential application for lignin modification: (1) lowering the lignin content, (2) incorporation of unique monomers into the lignin structure and (3) modification of plant cell wall proteins (Boerjan et al. 2003; Vanholme et al. 2010; Damm et al. 2016; Liu et al. 2018). The first strategy is accomplished through the increased integration of monolignols, which are usually present in lower quantities. For example, softwoods are known to have exclusively G units in their lignin layers. Applying this strategy would involve the engineering of S units because of the lower degree of their cross-linkages, as compared to G units (Boerjan et al. 2003). Additionally, decreasing the lignin content while increasing the cellulose content is another viable strategy. This strategy would greatly benefit the pulp and paper industries as they utilize large quantities of cellulose. These techniques serve to 'soften' the lignin structure, thus lessening the intensity of harsh pre-treatment processes that are usually needed to remove it. The addition of new genes (encoding unique monomers) into the plant genome disrupts the lignin assembly and contributes to the fragility of the barrier. Modifying cell wall polysaccharides can potentially lead to the facilitation of lignocellulose degradation without the requirement of extreme pre-treatments (Damm et al. 2016) and further, if suitably modified, could potentially lack the necessity of pre-treatment (Li et al. 2008).

6.2.4.2 Targets for Genetic Adjustment

Transgenic studies have been performed using the model plant, *Arabidopsis thaliana* (thale cress). In recent years, experimentation has also extended to numerous other organisms like pines, *Pinus alba*, *Pinus taeda* and *Pinus radiata* (Wagner et al. 2015; Edmunds et al. 2017); *Eucalyptus* trees (Sykes et al. 2015); poplar trees (*Populus* spp.); tobacco plants, *Nicotiana tabacum*; and grasses like maize (*Zea mays*) and alfalfa (*Medicago sativa*) (Vanholme et al. 2008; Weng et al. 2008; Chanoca et al. 2019). The targeted enzymes, for genetic adjustment, are those belonging to the monolignol pathway, namely, phenylalanine ammonia-lyase (PAL); cinnamate-4-hydroxylase (C4H); 4-coumarate:CoA ligase (4CL); hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase (HCT); *p*-coumarate 3-hydroxylase (C3H); caffeoyl-CoA *O*-methyltransferase (CCoAOMT); cinnamoyl-CoA reductase (CCR); and cinnamyl alcohol dehydrogenase (CAD) (Vanholme et al. 2008; Chanoca et al. 2019).

Extensive studies have demonstrated the possibility of engineering the lignin content of flora. The downregulation of enzymes HCT and C3H have resulted in plants with high H units, which are usually present in extremely low quantities in wild-type plants (Vanholme et al. 2008). The downregulation of enzymes ferulate 5-hydroxylase (F5H) and caffeic acid/5-hydroxyconiferaldehyde *O*-methyltransferase (COMT) leads to lignin comprising of G units and incorporation of 5-hydroxyconiferyl alcohol, respectively. However, overexpression of F5H results in plants with solely S units in lignin (Vanholme et al. 2008; Wagner et al. 2015). Modification of the C3H and C4H genes in *Eucalyptus urophylla* × *E. grandis* demonstrated enhanced saccharification accompanied by a decrease in the height of the plants (Sykes et al. 2015). A related study conducted on *Pinus tremula* and *P. alba*, targeting the CAD gene, also yielded plants with enhanced saccharification efficiency (Van Acker et al. 2017). A field test conducted with CCoAOMT downregulation in poplar, Beijing, demonstrated an increase in saccharification efficiency, while CCR downregulation in poplar, France, displayed increased pliability to kraft processing (Chanoca et al. 2019). The genetic studies conducted in the last 5 years have been tabulated (Table 6.2).

Although promising results have been obtained under laboratory (greenhouse) conditions, sometimes field reports don’t always reflect the same. The reason for this is that greenhouses lack the environmental parameters which could influence lignification, reversal of transgenic plants to wild type, impact of pathogen resistance, stress tolerance, etc. (Chanoca et al. 2019). For example, downregulation of 4CL in poplars grown in a greenhouse had lower lignin content than the same trees grown under field conditions (Xiang et al. 2017; Chanoca et al. 2019).

Even though there is much experimentation to be done, such studies provide proof of the viability of this method. At present, most of these studies aim to produce suitable feedstock for biofuel production. However, as the main ingredient, sugars, is required both for biofuels and for PHA production, collaborative processes can be investigated.

6.3 Direct Conversion of Lignocellulosic Agro-waste to PHA

Research on the direct conversion of lignocellulose to PHA has been undertaken over the years in an attempt to discover an organism with the potential to perform this feat in a singular bioprocessing step. The rationale is to eliminate the length of the process, thereby curbing excessive expenditure, energy inputs, sophisticated equipment, etc. (Govil et al. 2020). A marine isolate, *Oceanimonas doudoroffii*, was selected for PHA production for its ability to utilize lignin and lignin derivatives, as singular sources of carbon. Through analysis, it was revealed that lignin conversion to PHA was executed via sinapinic acid and syringic acid (Numata and Morisaki 2015). A study was conducted using agricultural waste for the production of PHA. Pineapple pieces and sugarcane waste were the agricultural carbon sources used by *Bacillus* spp. to produce PHA, yielding 1.86 g/L PHB with productivity 0.077 g/L/h (Suwannasing et al. 2015). A related study evaluated the potential of

Table 6.2 Summary of genetic adjustment studies conducted in the last 5 years (adapted from Chanoca et al. 2019)

Organism(s)	Target gene	Technique of modification	Growth	Lignin content	Composition of lignin	Remarks	References
Pinus alba × *P. grandidentata*	PMT	Overexpression	Greenhouse	Wild type	Wild-type S/G, incorporation of *p*-coumarate conjugates		Smith et al. (2015)
Eucalyptus urophylla × *E. grandis*	C3H	Antisense	Greenhouse	27% decrease	Decreased S/G, increased H units	Saccharification efficiency increased, height decreased	Sykes et al. (2015)
Eucalyptus urophylla × *E. grandis*	C4H	Antisense	Greenhouse	Decreased 36%	Decreased S/G	Saccharification efficiency increased, height decreased	Sykes et al. (2015)
Pinus radiata	F5H + COMT	Overexpression	TE cultures	N.D.	Incorporation of S units		Wagner et al. (2015)
Pinus tremula × *P. alba*	4CL1	CRISPR/Cas9 mutants	Greenhouse	23% decrease	Decreased S/G		Zhou et al. (2015)
Pinus tremula × *P. alba*	4CL2	CRISPR/Cas9 mutants	Greenhouse	Wild type	Wild-type S/G		Zhou et al. (2015)
Pinus tremula × *P. alba*	MOMT4	Overexpression	Greenhouse	15% decrease	Decreased S/G, decreased *p*-hydroxybenzoic acid	Saccharification efficiency increased	Cai et al. (2016)
Pinus taeda	4CL	Xylem-specific RNAi	Greenhouse	33% decrease	N.S.	Saccharification efficiency increased	Edmunds et al. (2017)

P. taeda	F5H + COMT + SAD/ CAD	Overexpression	Greenhouse	Wild type	Incorporation of S units		Edmunds et al. (2017)
Pinus alba × *P. grandidentata*	FMT	Overexpression	Greenhouse	N.S.	N.S.	Saccharification efficiency increased	Kim et al. (2017)
Pinus tremula × *P. alba*	CSE	Hairpin	Greenhouse	25% decrease	Increased H units, decreased S/G	Saccharification efficiency increased	Saleme et al. (2017)
Pinus tremula × *P. alba*	CAD	Hairpin	Greenhouse	10% decrease	Decreased S/G, increased sinapaldehyde	Saccharification efficiency increased	Van Acker et al. (2017)
P. nigra L. × *P. maximowiczii A*	4CL	Antisense	Greenhouse	47% decrease	Increased S/V		Xiang et al. (2017)
Populus alba × *P. grandidentata*	FMT	Overexpression	Greenhouse	Wild type	Wild-type S/G	Saccharification efficiency increased	Bhalla et al. (2018)
Pinus alba × *P. glandulosa*	C3H	Downregulation	Greenhouse	30% decrease	N.S.	Decrease in diameter of stem	Zhou et al. (2018)
Pinus alba × *P. glandulosa*	HCT	Downregulation	Greenhouse	20% decrease	N.S.	Decrease in diameter of stem	Zhou et al. (2018)
Oryza sativa L. spp. *japonica* cv. Nipponbare	CAD	Chemical examination of mutant	Greenhouse	N.S.	Increased S/G		Martin et al. (2019)

PMT *p*-coumaroyl-CoA monolignol transferase, *C3H* *p*-coumarate 3-hydroxylase, *C4H* cinnamate-4-hydroxylase, *F5H* ferulate 5-hydroxylase, *COMT* caffeic acid *O*-methyltransferase, *4CL* 4-coumarate:CoA ligase, *MOMT4* monolignol 4-*O*-methyltransferase, *CAD*/*SAD* cinnamyl alcohol dehydrogenase/sinapyl alcohol dehydrogenase, *FMT* feruloyl-CoA monolignol transferase, *CSE* caffeoyl shikimate esterase pineapple peel waste as feedstock for PHA production. *Ralstonia eutropha* was used as the PHA producer, and polymer analyses revealed the identity of the polymer, PHB (Vega-Castro et al. 2016).

Interestingly, studies evaluating the worth of seed-based feedstocks for PHA production were undertaken. Recently, a study was conducted using a medium containing 1.5% (v/v) rubber seed oil for PHA production by *Bacillus cereus* and yielded 2.16 g/L PHA at 72 h fermentation (Kynadi and Suchithra 2017). In another study, a dual feedstock of date seed oil and date molasses was utilized for copolymer production by *Cupriavidus necator* H16 Re2058/pCB113. The copolymer formed was poly-β-hydroxybutyrate-co-β-hydroxyhexanoate [P(3HB-co-3HHx) or PHBH] with molecular weight ranging from 500 to 690 kDa and 28 mol% 3HHx units. The dual combination resulted in PHA accumulation of 80% as compared to 28% PHB accumulation from date molasses (Purama et al. 2018). Thus, seed-based feedstocks are an untapped source of sugars and should be investigated further.

The employment of microbial co-cultures and consortia has been investigated and hold promise for effective PHA production. A study was conducted to assess the conversion of plant and algae biomass by *Saccharophagus degradans* (formerly *Microbulbifer degradans*) 2-40, *Bacillus cereus* and co-culture of both. The results revealed that the co-culture yielded a higher PHA accumulation of 34.5% than the individual cultures (Sawant et al. 2017). Interestingly, *S. degradans* 2-40 was proven to attach and degrade the red seaweed *Gelidium amansii* for the production of PHA, yielding PHA accumulation of 17–27% (Sawant et al. 2018). In a separate study, co-cultures of *Ralstonia eutropha* H16 and *Ralstonia eutropha* 5119 were co-cultured with *Bacillus subtilis* and *Bacillus amyloliquefaciens* (sucrose-metabolizing organisms). Co-culture of *R. eutropha* 5119 and *Bacillus subtilis* resulted in 45% PHA accumulation, which was optimized to yield 66% accumulation. Polymer analyses revealed the polymer to be P(3HB-co-3HV) consisting of 16 mol% 3HV (Bhatia et al. 2018).

Although nature has provided potent PHA-producing bacteria, numerous scientists have been attempting to engineer bacterial PHA producers with capabilities to act directly on lignocellulose, for the production of PHA in a singular bioprocessing step. In a recently published report, *Pseudomonas putida* KT2440 was subjected to numerous genetic modifications to improve the accumulation of medium-chain-length (mcl) PHA from *p*-coumaric acid (a monomer of lignin) and lignin. The modifications carried out were (1) elimination of the mcl PHA depolymerization gene (*phaZ*); (2) decrease in direction of mcl PHA pathway intermediates to fatty acid degradation via deletion of enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase (*fadB*) and 3-ketoacyl-CoA thiolase (*fadA*) genes; and (3) increase of carbon from fatty acid chain elongation to mcl PHA production through the integration of the hydroxyacyl-ACP thiolase (*phaG*), the hydroxyacyl-CoA synthase (*alkK*) and the two PHA polymerase (*phaC1* and *phaC2*) genes. This resulted in the strain, *P. putida* AG2162, which yielded a 53% and 200% increase in mcl PHA titre (g/L) and yield (g mcl PHA/g dry cell weight), respectively, from *p*-coumaric acid. Additionally, this was accompanied by an increase of 20% mcl PHA titre (g/L) and 100% yield (g mcl PHA/g dry cell weight) from lignin, when compared with the wild-type strain (Salvachúa et al. 2020). A similar study was conducted using the same strain, *Pseudomonas putida* KT2440, wherein the CRISPR/Cas9n-λ-Red system was used to edit the organism's genome to include nine genes responsible for ferulic acid catabolism and production of PHA. The resultant strain, KTc9n20, produced 270 mg/L PHA from a system with 20 mM ferulic acid (Zhou et al. 2020). Ferulic acid is an intermediate phenolic metabolite formed during lignocellulose pre-treatment and biodegradation. This method appears to be very promising for utilization as an alternate for PHA production. However, PHA yields still need to be enhanced through further experimentation and optimization.

An extensive review of literature, over the past two decades, has postulated the benefits and sustainability of establishing biorefineries in close proximity to industries, thereby creating opportunities for utilizing waste streams and thus reducing pollution and disposal issues while generating additional income (Koller et al. 2010; Jiang et al. 2016; Dietrich et al. 2019). However, minimal studies have investigated the viability of such an endeavour. Recently, an interesting study was conducted which evaluated the economic efficiency of installing a biorefinery attached to a sugar mill for the simultaneous production of poly-β-hydroxybutyrate (PHB), succinic acid and electricity from sugarcane bagasse and mill trash lignocellulosics. Of the four scenarios considered, the most viable arrangement was found to be the production of PHB from quarter of the fermentable sugar stream coupled with succinic acid production from the remaining three quarters of the sugar stream. This resulted in US$477.2 million net present value coupled with 24.1% internal rate of return (IRR). Thus, such a proposition affords a sustainable investment opportunity for sugar mills (Nieder-Heitmann et al. 2019).

6.4 Conclusion

PHA has the desirable characteristics, which make it an attractive alternative to non-biodegradable petroleum and oil-based plastics. It is completely biodegradable and biocompatible with UV resistance, thermoplasticity and non-toxicity. Unfortunately, the process of PHA production is costly due to the high expense of raw materials coupled with low efficiency. Thus, scientists have developed techniques to employ lignocellulose, the most copious carbon cache, as an inexpensive feedstock for PHA production. Although there are many issues involved with the usage of lignocellulose, various pre-treatment methods have been developed to overcome these obstacles. Of the multitude of pre-treatment methods, techniques like biological pre-treatment, genetic manipulation, etc. have begun to catch the attention of scientists. Each pre-treatment has its profits and losses; however, a universal pre-treatment method, which can suit all types of biomass (agricultural residues, softwoods, hardwoods, etc.), is yet to be developed. In the last 5 years, several studies have been conducted to evaluate different lignocellulosic agro-wastes for the production of PHA. The goal of sustainability coupled with minimal capital and energy is the driving force behind these studies. Many studies have shown promising results. However, this process is still in its infancy and requires further investigation if it is to be established and commercialized.

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7

Fruit Waste as Sustainable Resources for Polyhydroxyalkanoate (PHA) Production

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Abstract

Production of polyhydroxyalkanoate (PHA) using commercially available carbon sources like glucose or sucrose makes the bioprocess economically nonviable, thereby hindering its commercialization. As an alternative to this issue, inexpensive and easily available agro-industrial wastes are now being exploited as feedstock for PHA production. Fruit wastes are generally discarded as they are considered to be the non-product leftovers which do not have any economic value when compared with the cost of their collection and recovery steps for reuse. But through the use of appropriate technological applications, these wastes can be converted to valuable by-products, which can increase the value of the products much higher than the cost associated with recovery steps. By recycling and reprocessing the fruit wastes, they can be channeled into many applications, and thereby the amount of fruit wastes discharged into the environment can be completely reduced along with their detrimental effects. Large amounts of fruit wastes are produced by fruit-based industries. The waste products can be both solids and liquids, and these wastes are of high nutritional and biomass values for microorganism; thus their addition to waterbodies can make them highly polluted (high BOD or COD). These fruit-based wastes still have a promising potential for bioconversion into products of commercial importance or can be successfully exploited as cheap raw materials for industrial production of commercially important metabolites. This chapter deals with the strategies for production of

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PHA from fruit waste substrates, extraction and characterization of PHA, and their applications in diverse sectors.

Keywords

Polyhydroxyalkanoates · Biopolymers · Downstream processing · Fruit waste · Biodegradability

7.1 Introduction

Plastic has gained importance in day-to-day life of human beings, mainly because of its durability, lightweight, low manufacturing cost, and the property to resist physical stress and chemical and biological degradations. The most important property of plastic is that its chemical structure can be engineered to give us the desired shapes with the desired property, such as packaging materials and disposable goods (Du and Yu 2002). Plastics are generally multichains of polyethylene, polypropylene, polystyrene, and polyvinyl chloride and their other forms. The main disadvantage of plastic, its "non-biodegradability," is the main fear haunting earth and its environment. The monomers of plastics are larger in size making the microorganism to have a lesser effect on their degradation, making them in the top category of xenobiotics (Brandl et al. 1990; Reddy et al. 2003). The quantity of these plastics are exponentially increasing in the planet; early in the 1950s, the plastic production was 2 million tonnes per year, and in 2015, it has crossed over 385 million tonnes per year, and the majority of these plastics are disposed of into the environment (Ritchie and Roser 2018). Due to their existence and accumulation over centuries, they are imposing a huge strain on the environment (Albertsson et al. 1987). New enzymes which are capable of degrading these pollutants are yet to be formed, and the built-in mechanism of nature is unable to degrade these synthetic polymers (Mueller 2006).

The plastics dumped in the environment are affecting waterbodies, forest, and even cities. The waterbodies and forests filled with plastics will create a hostile environment to the organisms living in them and can eventually lead to eutrophication in the case of lakes and ponds (Ojumu et al. 2004). The common measures of plastic disposal routinely followed include landfilling, incineration, and recycling. An estimate of plastic disposal highlights that 55% of the plastic produced are being disposed of and littered, which will be blown to the sea and eventually hit the marine life hard (Moore 2008). Twenty-five percent of the remaining are incinerated, which releases a lot of gases like carbon dioxide, carbon monoxide, furans, dioxins, hydrogen chloride, hydrogen cyanide, nitrogen oxides, and benzopyrene which can disturb their optimal levels in the atmosphere and can result in an unhealthy and corrosive environment (Jayasekara et al. 2005). The remaining 20% are recycled, but this in turn reduces the quality of the plastic by altering its chemical structure or material properties. The recycling cost for plastic is high, and the application range of recycled plastics is very much less as compared to virgin plastics available in the market. This makes the manufacturers prefer virgin plastics over recycled ones. The one-time usage and steady existence of plastics increase their rate of accumulation in the environment and the resulting adverse effects (Mohanty et al. 2002).

7.2 Bioplastics

Bioplastics also known as biodegradable polymers are the polymers which can be degraded by the activity of common microorganisms. Bioplastics have the capability to replace the petroleum-based synthetic plastics which we use in day-to-day life. These polymers are synthesized by a range of organisms under different environmental and nutritional conditions, as storage molecules (stored in mobile, amorphous, liquid granules) to cope up with a stressed environment. Based on the source, bioplastics are classified into different types like plant-based (starch, lignin, gluten, cellulose, etc.), animal-based (chitin and chitosan), and microbial-based (PHA, xanthan, pullulan, and chitin) (Al Ali AlMaadeed et al. 2020). The bioplastics can be degraded under any condition at any place where microorganisms reside like landfills, composts, or sewage treatment plants. The main components which are released during the degradation are carbon dioxide, water, and humic substances, which don't cause any harm to the environment (Siracusa 2019).

7.3 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are one of the most focused groups among biodegradable plastics. These are polyesters of hydroxyalkanoates and are produced and accumulated as intracellular granules in numerous prokaryotes as an energy source under stressed conditions (usually with excess carbon coupled with limitation of nitrogen or any other essential nutrients). Other than energy storage, these PHA granules are known to enhance the organisms' robustness against various stress factors like maintaining cell integrity during an osmotic shock (Sedlacek et al. 2019). The bioplastics in the PHA family are the only class of 100% biodegradable polymers, exclusively synthesized by microorganisms, and more than 80% of PHA accumulation (with respect to cell dry weight) was detected in different bacteria (Lee 1996). Therefore, it can be completely degraded easily into the simplest compounds which are of no harm to the environment by the accumulating strains itself with their depolymerase enzyme. PHAs can be degraded under aerobic and anaerobic conditions unlike many other bioplastics, and they do not need any catalysts or additives to step up their degradation. The majorly studied class of PHA includes poly-beta-hydroxybutyric acid (PHB) and poly-beta-hydroxyvaleric acid (PBHV). PHB was discovered first in bacteria, and it occurs as various copolymers. These copolymers are known to have better mechanical and film-forming abilities like the low-density polyethylene.

7.3.1 Chemical Structure of PHA

PHAs are monomers of hydroxyalkanoic acids which are linked by ester bonds, and the length of PHA polymer (*n*-value) can be more than 3000 monomers. Based on the producing organism, PHA length can differ, and the length of the (R)-pending group also has a role in determining molecule's length (Fig. 7.1). The most commonly occurring PHA is PHB, and hence they are the widely studied class of PHAs. The mechanical properties of PHA are completely based on the monomer unit, and it is reported that more than 120 monomer units of PHA with a wide variety of (R)-pending groups have been discovered. The hydroxyl group and carboxyl group of adjacent PHA monomers form the ester bond and hence the polymer. (R)-3-hydroxyalkanoic acid is the major molecule present in the PHA monomer, and a wide variety of (R)-pending groups varying from (C1) methyl to (C8) tridecyl were observed (Table 7.1). In fact, the wide range of application of PHAs comes from the huge variability of functional groups, length of the chain, and number of unsaturated bonds. The chemical nature of the synthesized PHA also depends on the microbial

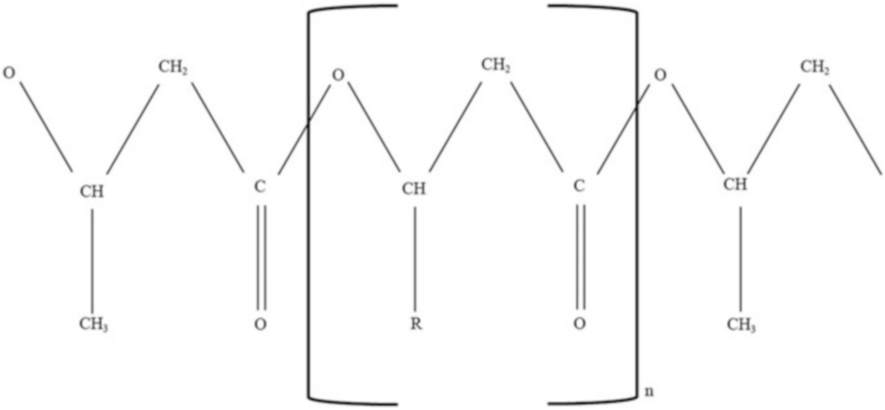


Fig. 7.1 Basic structure of PHA

Table 7.1 Classification of PHAs based on the R-group

R-group	Abbreviation	Polymer name
Hydrogen = H	PHP	Poly(3-hydroxypropionate)
Methyl = CH_3	PHB	Poly(3-hydroxybutyrate)
Ethyl = CH_2CH_3	PHV	Poly(3-hydroxyvalerate)
Propyl = $(CH_2)_2CH_3$	PHHx	Poly(3-hydroxyhexanoate)
Butyl = $(CH_2)_3CH_3$	PHH	Poly(3-hydroxyheptanoate)
Penyl = $(CH_2)_4CH_3$	PHO	Poly(3-hydroxyoctanoate)
Hexyl = $(CH_2)_5CH_3$	PHN	Poly(3-hydroxynonanoate)
Heptyl = $(CH_2)_6CH_3$	PHD	Poly(3-hydroxydecanoate)
Octyl = $(CH_2)_7CH_3$	PHUD	Poly(3-hydroxyundecanoate)
Octyl = $(CH_2)_7CH_3$	PHDD	Poly(3-hydroxydodecanoate)

strain producing it, the type of substrate (carbon source) used, and its relative quality and quantity (Liebergesell et al. 1993). Based on the carbon chain length of the monomeric units, PHAs can be categorized into the following three groups (Volova 2004):

1. Short-chain-length PHA (scl-PHA)—Monomer chain has three to five carbon atoms.
2. Medium-chain-length PHA (mcl-PHA)—Monomer unit consists of 6–14 carbon atoms.
3. Long-chain-length PHA (lc1-PHA)—Monomer unit with 17–18 carbon atoms.

A stiff material with high crystallinity, high tensile modulus, and long elongation at break is obtained when the small side chains are methyl or ethyl groups of scl-PHA. These are brittle with high melting temperature around 170 °C, which may result in polymer decomposition and therefore limits the applicability of this homopolymer (Madison and Huisman 1999). The introduction of 3-hydroxyvalerate into PHB results in a copolymer of poly-3-hydroxybutyrate-co-3-hydroxyvalerate, which is less stiff and brittle than the normal PHB, thus expanding its application range. As the side chain length increases, the polymer formed becomes more elastic.

7.3.2 Enzymatic Synthesis of PHA

PHA biosynthesis is catalyzed by mainly three groups of enzymes (Madison and Huisman 1999):

β-Ketothiolase: These are homo-tetrameric enzymes which have molecular mass of 160–190 kDa. It catalyzes the formation of C-C bonding and the N-terminal amino acid sequence. β-Ketothiolase gets inhibited by coenzyme A.

Acetoacetyl-CoA Reductase: They are homo-tetramer with identical subunits, and the molecular weight ranges between 85 and 140 kDa. They involve in PHA synthesis and catalyze oxidation-reduction reaction between hydroxybutyrate and acetoacetate.

PHA Synthase: They act on hydroxyalkanoate monomers and form the ester bonds to produce the PHA polymer. The substrates for this group of enzymes are coenzyme A (CoA) thioesters of hydroxyalkanoic acids (HAs) and release CoA while catalyzing the polymerization of HAs into PHA. Depending on the microbial strain, their molecular weight varies.

7.3.3 Biosynthetic Pathways for PHA Production

The microorganisms utilize the carbon source in order to attain energy, and the sugar molecules are subjected to glycolysis which gives out pyruvate molecules. These pyruvate molecules are converted to acetyl-CoA and then channeled to the Krebs cycle as the coenzyme A released during the Krebs cycle in a regular microbial growth with sufficient nutrition will inhibit the activity of β-ketothiolase enzyme and suppresses the PHA biosynthetic pathway. When we induce a low-nutrient stress, the acetyl-CoA will not enter into the Krebs cycle and will get accumulated; these accumulated acetyl-CoA will be used up to synthesize PHA (Liebergesell et al. 1993). By doing intense study on the groups of bacteria producing PHA, four different biosynthetic pathways for PHA production are reported.

7.3.3.1 PHA Biosynthetic Pathway I

This is the most commonly observed PHA biosynthetic pathway in PHA-producing bacteria (Liebergesell et al. 1993). This pathway is studied well in *Cupriavidus necator* (Fig. 7.2). There are three genes responsible for this pathway: *pha* A, *pha* B, and *pha* C. *pha* A gene codes for β-ketoacyl-CoA thiolase which catalyzes the condensation reaction between two acetyl-CoA molecules to generate acetoacetyl-CoA. This acetoacetyl-CoA is reduced by NADPH-dependent acetoacetyl-CoA dehydrogenase, encoded by *pha* B gene, to (R)-3-hydroxybutyrate monomer. Enzyme PHA synthase encoded by *pha* C gene will then carry out the polymerization between the monomer units by forming the ester linkage (Anderson and Dawes 1990).

7.3.3.2 PHA Biosynthetic Pathway II

This pathway is evident in *Rhodopseudomonas rubrum*; PHA is produced by β-oxidation of fatty acids (Fig. 7.3). The initial step of this pathway is the same as pathway I, where β-ketoacyl-CoA thiolase condenses two acetyl-CoA molecules and forms acetoacetyl-CoA. In the next step, Enol-CoA is converted into (S)-3-hydroxyacyl-CoA by the action of Enol-CoA hydratase enzyme and then into to 3-Ketoacyl-CoA with the help of 3-Ketoacyl-CoA dehydrogenase. This Enol-CoA can

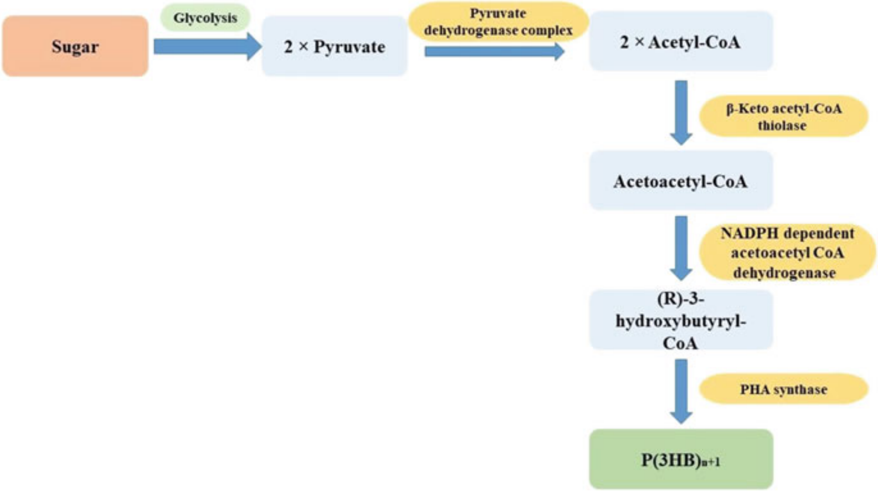


Fig. 7.2 PHA biosynthesis pathway I

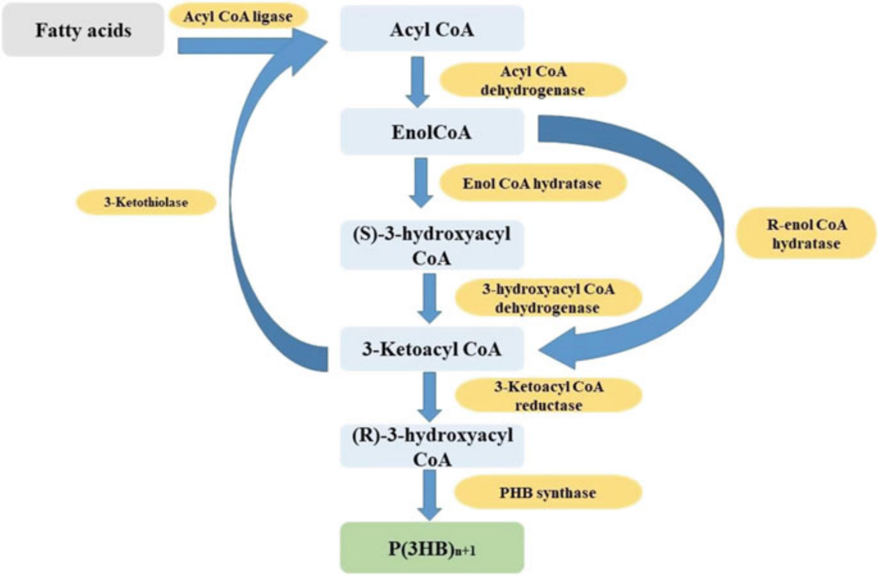


Fig. 7.3 PHA biosynthesis pathway II

also be directly converted into 3-Ketoacyl-CoA by the action of R-enol CoA hydratase. 3-Ketoacyl-CoA can also carry out the feedback inhibition on the first step with the help of 3-Ketothiolase enzyme. Finally, (R)-3-hydroxyacyl-CoA is formed from 3-Ketoacyl-CoA by the action of 3-Ketoacyl-CoA reductase (Khanna and Srivastava 2005). The PHA polymerization happens by the action of PHB synthase.

7.3.3.3 PHA Biosynthetic Pathway III

This pathway was majorly studied in *Pseudomonas oleovorans* of *Pseudomonas* group I type (Fig. 7.4). This pathway is almost identical to PHA biosynthetic pathway II, except mcl-PHA is produced at the end of the pathway and the PHA production is happening through β-oxidation of alkanes, alkanols, and alkanoic acids (Naik et al. 2008). Initially the alkenes and alkanes are converted into fatty acids by terminal oxidation, and these fatty acid molecules are subjected to β-oxidation. During the β-oxidation, the compounds enol-CoA and 3-ketoacyl-CoA are formed as intermediates. The enzyme R-enol-CoA hydratase converts enol-CoA to (R)-3-hydroxyacyl-COA, and the 3-ketoacyl reductase reduces the 3-ketoacyl-CoA to (R)-3-hydroxyacyl-COA. The PHA production from (R)-3-hydroxyacyl-COA is catalyzed by PHA synthase.

7.3.3.4 PHA Biosynthetic Pathway IV

Pathway IV is studied in *Pseudomonas* group II type (Fig. 7.5), and the de novo fatty acid synthesis pathway is used to produce PHA which involves copolymer mcl-PHA

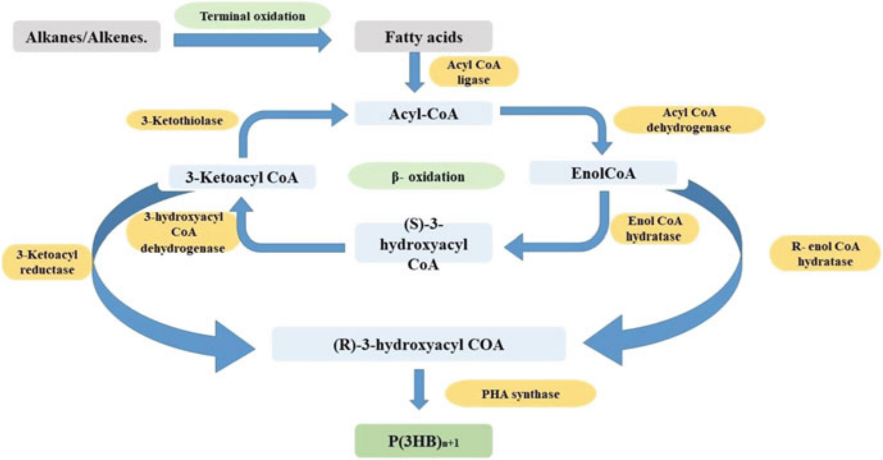


Fig. 7.4 PHA biosynthesis pathway III

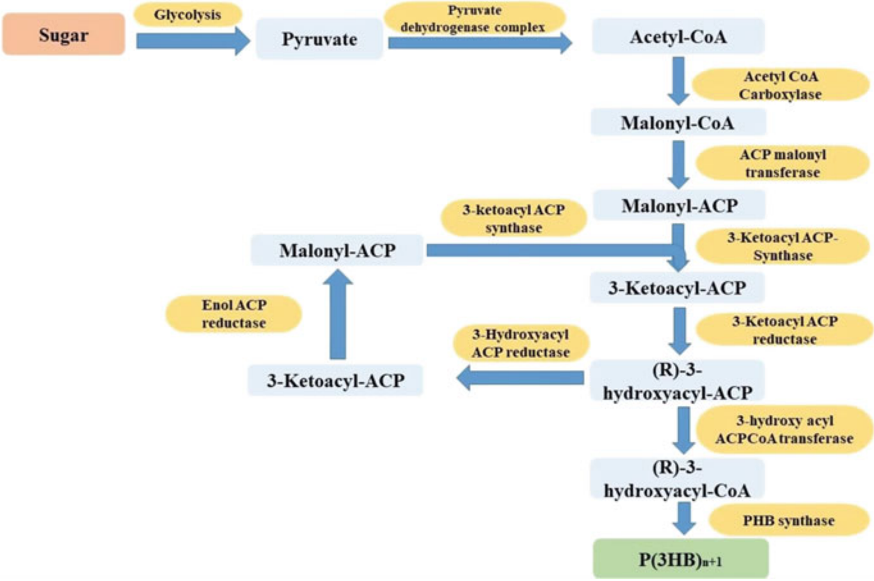


Fig. 7.5 PHA biosynthesis pathway IV

synthesis from acetyl-CoA (Naik et al. 2008). The (R)-3-hydroxyacyl-ACP formed during the de novo pathway will be transformed into (R)-3-hydroxyacyl-CoA by the enzyme 3-hydroxyacyl-ACP-CoA transferase. The (R)-3-hydroxyacyl-CoA is converted to PHA polymer by the enzyme PHA synthase.

7.3.4 Properties of PHAs

7.3.4.1 Physical Properties

The physical properties of PHAs are much similar to that of polypropylene that constitutes the conventional plastics. The PHA molecules can be made of more than 30,000 monomers, and they are lipophilic in nature and thus immiscible in water. The solubility of PHA depends on its chemical composition. Generally, PHAs are soluble in chlorinated organic solvents like methylene chloride, chloroform, 1,2-dichloroethane, and propylene carbonate. These polymers are optically active and piezoelectric. Based on the microbe producing the PHA, their molecular weight ranges from 2×10^5 to 3×10^6 Da.

7.3.4.2 Chemical Properties

PHAs are optically active polyesters made up of (R)-3-hydroxyalkanoic acid (HA) monomer units. Due to the stereospecificity of the PHA synthase (polymerizing enzyme), all the 3-HA monomers in the PHA polymer are in R configuration. The side chains of the polymer have considerable variations in length and composition, due to which a wide variety of applications is possible for PHA. The C3 alkyl group in PHAs may vary from methyl to tridecyl components and this alkyl chain can be saturated, unsaturated, epoxidized, aromatic, halogenated, or branched monomers.

7.3.4.3 Mechanical Properties

The mechanical and thermal properties of PHAs are remarkably similar to the plastics synthesized from petroleum. Due to their promising property of biodegradability, they have the capacity to replace the use of plastics from many of their applications. The mechanical properties of PHA also depend on the monomeric units which make up the polymer. The mcl-PHA polymers are semi-crystalline elastomers, and the scl-PHAs have similar properties with thermoplastics. The properties like low tensile strength, low Tm (melting temperature), and high extension to break make them biodegradable rubber substitutes. PHB which is one of the scl-PHAs has a very identical melting temperature (Tm) and degree of crystallinity to that of polypropylene (PP), but PHBs are brittle and stiffer than PP. But studies proved that copolymerization of PHBs with hydroxyvalerate (HV) monomer units increased the toughness and reduced the stiffness. This opens the commercial application of the PHB.

7.3.4.4 Biological Properties

Biodegradability and biocompatibility account for the biological properties of PHA. Biodegradability is the change in the physical and chemical forms of the PHA polymer by the action of environmental, chemical, or biological agents (Umesh and Thazeem 2019). The PHA molecules can be degraded completely by microbial enzymes into carbon dioxide and water, aerobically. PHAs are degraded to methane and water anaerobically (Ojumu et al. 2004). Biocompatibility is the scale of efficacy when the biological application is added into a system. For PHA, their biocompatibility opens a wide scope in the medical field, as there are no toxic compounds produced after the degradation. P(3HB) is found to be more compatible for medical use, as 3HB is present in blood and it is also a constituent of the eukaryotic cell envelope (Reusch 2000). Copolymers of PHB and PHBV and copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate are administered in animal models, and these polymers are found to be biocompatible in a variety of host systems (Misra et al. 2006). PHAs have many applications in medicine; it is used in vascular system devices (vascular grafts, heart valves), controlled drug delivery (via macro- and nanocarriers), wound management (skin substitutes, sutures), urological stents, orthopedic (screws, bone graft substitutes, scaffolds for cartilage engineering), etc. (Wang et al. 2013).

7.3.5 Applications of PHA

The issues with synthetic plastic are growing day by day, so the world is turning its eyes on research, production, and commercialization of biodegradable plastics. Among biodegradable plastics, PHA is a powerful candidate which can mimic qualities of petroleum-based plastics, like the thermoplastic properties and moldability, which expands its applications in various areas (Arun et al. 2009). The application area of PHAs is divided into four major domains (Holmes 1985): medical and pharmaceutical application, industrial application, agricultural application, and other applications.

7.3.5.1 Applications of PHA in the Medical and Pharmaceutical Fields

The ability to degrade completely leaving no toxic materials makes them fit for biomedical applications (Sun et al. 2007). The degradation of 3HB produces common metabolic products like carbon dioxide and water which makes the molecule biocompatible for its biomedical uses (Sevastianov et al. 2003). The PHA polymer can be conjugated or combined with other polymers, enzymes, and inorganic materials to improve its biocompatibility and mechanical properties. Intense researches are going on using many PHAs and their copolymers for studying applications like their use as sutures, orthopedic tools, repair patches, adhesion barriers, articular cartilage, repair devices, nerve guides, bone plates, osteosynthetic materials, cardiological stents, wound dressings, and tissue engineering materials. PHA-HA (hydroxyapatite) blend is used in the healing process during bone injuries by serving as hard tissue support, due to its very close structure and mechanical strength to the human bones (Chen et al. 2005).

The properties of surface erosion and biocompatibility make PHAs a potential choice in drug carriers, and hence they are used in sustained drug release (Koosha et al. 1989), and they are also used as subcutaneous implants, compressed tablets, and intravenous micro-particulate carriers. Certain PHBs and their copolymers are used to make films of moisture in sanitary towels and nappies and paramedical films for hospital use (Hocking and Marchessault 1994). PHBs are also used as surgical glove dusting powders that can serve as lubricant in surgeon's gloves as they are completely biocompatible (Holmes 1985). Another biomedical application of PHA is that amphiphilic PHA copolymer graft is used as a blood-contacting device because of its high compatibility with blood (Hazer and Steinbüchel 2007).

7.3.5.2 Industrial Applications

The property of thermoplasticity gives a wide field of application for PHAs in the industrial field. The main use of PHAs directly comes in the packaging and coating sectors (Bucci et al. 2005). PHAs' gas barrier property is precisely suited for the manufacture of food packaging and plastic bottles and can be used to make milk packages (Hocking and Marchessault 1994). Plastic materials tend to float in water, but the PHA bioplastics sink to the bottom of water, which will enhance the biodegradation (Luzier 1992). PHB-co-PHHx copolymer are reported to be used in the development of nonwovens, flushable benders, flexible packaging, thermoplastic items, synthetic paper, and medical devices (Chen et al. 2001), and the copolymer PHB-co-PHO is approved by FDA (Food and Drug Administration) to use as an additive in food ingredients. PHA polymers are also used to make gas lighters, keyboards, headphones, microphones, and loud speakers and used in ultrasound therapy due to their piezoelectric properties (Babel et al. 1990).

7.3.5.3 Agricultural Applications

PHAs are very useful in the agricultural sector (Philip et al. 2007); it is used for encapsulation of fertilizers and seed and as mulch films (Hocking and Marchessault 1994). PHAs with bacterial inoculants have promising nitrogen fixation abilities (Hocking and Marchessault 1994; Dobbelaere et al. 2001), and P3HB-co-HV copolymer is used for controlled release of insecticides and pesticides into the field.

7.3.5.4 Other Applications

PHA was reported to have many other applications as well. One of the important applications is the use of PHA in packaging of food and other items. PHA is proven to be a substitute for plastic; hence it can be easily used for packing of materials, and the plastic usage in this sector can be reduced. It was reported that when PHA is incorporated into aquatic feeds, the survival rates, immunity, and body weight gain of larvae and adults increased (Defoirdt et al. 2011; Semova et al. 2012). This proves that PHA can be used as substituents for antibiotics and growth enhancers in aquaculture. Other important applications are self-degrading surgical sutures, scaffold for tissue generation, containers for cosmetics and films for recovery after surgery and neuronal regeneration, and stents (Grigore et al. 2019). Some other important applications which are still under study are syringes, masks, and bandages using PHA. The fabrication of PHA sheets with antimicrobial compounds incorporated to it is also gaining importance (Umesh and Preethi 2017). These products which are existing in the market can be used only once; after use, they are becoming a headache as they are non-biodegradable, and once biodegradable PHAs are used to build them, this issue can be effectively resolved.

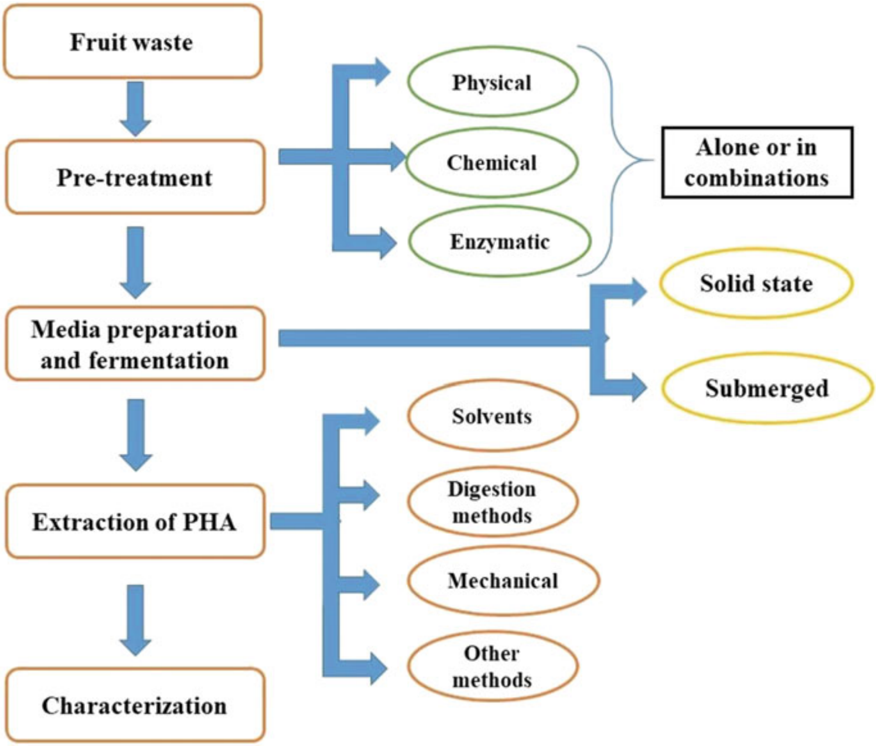


Fig. 7.6 Schematic outline of PHA production process using fruit waste

7.4 Fermentative Strategies for PHA Production from Fruit Waste

The methodology of PHA production using fruit waste involves their bioconversion into fermentable substrate for bacterial growth that leads to the accumulation of intracellular PHA granules (Preethi and Vineetha 2015). Although the composition of various fruits shows considerable variation from each other, any fruit that has an appreciable amount of carbohydrates can be used for the production of PHA (Umesh and Basheer 2018). The basic strategy involves a pretreatment methodology to release complex sugars into the fermentation medium followed by its hydrolysis to release fermentable sugars that can be easily utilized by the production strain. These processes can be achieved by a combination of physicochemical and enzymatic treatment procedures (Preethi et al. 2017). The selection of a particular method for substrate preparation readily depends on factors such as the nature of substrate (fruit waste) employed, the type and properties of the production strain, the overall economics of production, and the fermentation volume (Umesh and Basheer 2018). A basic outline of the PHA production process from fruit waste is illustrated in Fig. 7.6.

Another key factor to be considered is the possibility of bacterial growth inhibition or reduction due to the presence of active metabolites like phenolics in fruit waste. This may prove lethal to the production strain or sometimes affects their biosynthetic pathways leading to an inhibition or reduction in PHA production. Often these problems can be overruled by inactivation of these compounds prior to fermentation or using a consortium of microbes that makes the environment favorable for the otherwise susceptible bacteria. PHA fermentation from fruit waste can be done either through solid-state fermentation or submerged fermentation. As per the literature, the yield from solid-state fermentation is generally less as compared to a submerged fermentation (Castilho et al. 2009). In both the methods, addition of trace elements and their salts is done to support the bacterial growth and PHA accumulation. The bacteria inoculated into the fruit waste-based media consume the fermentable sugars and tune them to any of the PHA fermentation pathway (described in the above section) to sequentially convert them into PHA molecules. Careful monitoring of the production process and levels of bioprocess variables has a crucial role in the success of PHA production from fruit waste (Umesh et al. 2018). Deviation in proper monitoring and extraction within stipulated time may lead to the degradation of intracellular PHA by the same production strain through the synthesis of PHA depolymerase enzyme. The degraded PHA molecules are then utilized by the bacterial cell for its energy need. A detailed account of PHA production from fruit waste is depicted in Table 7.2.

7.5 Extraction of PHA

The cost of PHA production is mainly influenced by the downstream process that limits its application in a commercial scale (Jacquel et al. 2008). Novel cost-efficient methods to separate and purify PHA are yet under studies. A successful extraction system used in the process should never interfere with chemical and physical properties of PHA. The PHA extraction generally happens in two steps: cell pretreatment and lysis followed by separation or recovery of PHA from all the cell components. For cell disruption to happen, the cells are subjected to pretreatment. The most common pretreatments are applying heat, applying alkali or their salts, and freezing/thawing cycles. The aim of pretreatment is to either make the cell wall and membrane weak or to completely disrupt the cells, so that the extraction happens effectively. For PHA recovery, a variety of methods starting from old techniques like solvent extraction and enzymatic digestion to the modern techniques like floatation are available (Kunasundari and Sudesh 2011; Madkour et al. 2013).

7.5.1 Solvent Extraction

Lemoigne was the first to report PHA recovery by using solvent extraction technique (Lemoigne 1926). This extraction was done from *Bacillus* spp. using hot alcohol followed by purification using chloroform and diethyl ether. In general, chlorinated

Table 7.2 PHA production from different types of fruit waste

Fruit waste	Strain employed	PHA yield	References
Solaris grape pomace	*Pseudomonas resinovorans*	21.3 g/L	Follonier et al. (2014)
Apricot pomace	*Pseudomonas resinovorans*	1.4 g/L	Follonier et al. (2014)
Orange peel hydrolysate	*Bacillus subtilis* NCDC0671	5.09 g/L	Umesh et al. (2018)
Canned pineapple industry residue	*Cupriavidus necator* strain A-04	0.160 g/(L h)	Sukruansuwan and Napathorn (2018)
Banana pulp and peel	*Burkholderia sacchari* IPT101	31.5 kg/tonne of substrate	Naranjo et al. (2014)
Pineapple peel solution	*Bacillus drentensis* strain BP17	5.55 g/L	Penkhrue et al. (2020)
Pineapple peel waste	*Ralstonia eutropha* ATCC 17697	44 mg/100 g of substrate	Vega-Castro et al. (2016)
Oil palm empty fruit bunch	*Bacillus cereus suaeda* B-001	20 g/L	Yustinah et al. (2019)
Jackfruit seed hydrolysate	*Bacillus sphaericus* NCIM 5149	2.2 g/L	Ramadas et al. (2010)
Banana peels	*Zobellella* sp. DD5	1.13 g/L	Maity et al. (2020)
Papaya peels	*Bacillus subtilis* NCDC0671	4.2 g/L	Umesh et al. (2017)
Cantaloupe waste extract	*Bacillus megaterium* TISTR 1814	1.1 g/L	Rehman et al. (2020)
Pineapple cannery wastes	*Bacillus* sp. SV13	0.40 g/L	Suwannasing et al. (2015)
Jackfruit seed powder	*Bacillus* sp.	18 µg/mL	Mekala et al. (2011)
Apple pulp waste	Co-culture of *Cupriavidus necator* DSM 428 and *Pseudomonas citronellolis* NRRL B-2504	52% cell dry weight (CDW)	Rebocho et al. (2020)
Hydrolyzed citrus pulp	*Bacillus* sp. strain COL1/A6	54.6% CDW	Santimano et al. (2009)
Apple pulp waste	*Pseudomonas citronellolis* NRRL B-2504	30% CDW	Rebocho et al. (2019)

hydrocarbons and cyclic carbonates are used to dissolve the PHA molecules. The most common chlorinated hydrocarbons used are chloroform and 1,2-dichloroethane, and the common cyclic carbonate is 1,2-propylene carbonate (Lafferty and Heinzle 1980; Ramsay et al. 1994). For the extraction of special types of PHA, other classes of solvents are used, and for dissolving mcl-PHA, acetone (a ketone) is used (Jiang et al. 2006). The dissolved PHA can be extracted by evaporating the solvent or by precipitating the PHA molecules. The precipitation of dissolved PHA from this solvent is done by using anti-solvents; these anti-solvents are liquid in which the PHA molecules are not soluble. Addition of methanol in excess into the PHA dissolved solvent mixture will cause the PHA to precipitate out (Hocking and Marchessault 1994). It was also reported that water can be used for precipitating PHA from a methylene chloride solvent (Hanggi 1990); this finding can significantly reduce the cost of PHA production. The precipitated PHA can be separated by centrifugation techniques or by filtration (Umesh and Preethi 2017). Studies have found out that the solvent extraction method can reduce the toxic substances which can be synthesized by bacteria along with PHA. The problem related to the use of solvents for extraction is that the majority of solvents and anti-solvents are costly and they have a harmful effect on the environment and researchers using them.

7.5.2 Extraction by Digestion

The solvent extraction method facilitates the separation of PHA by dissolving it; in the digestion method, the cellular materials are digested either by using different chemicals or enzymes.

7.5.2.1 Chemical Digestion

Due to the availability of a variety of chemicals and knowledge about their interactions with the cellular components, the chemical digestion method is used more frequently than the enzymatic digestion methods. This method aims at solubilizing the non-PHA cellular masses; the most common chemicals used for the method are sodium hypochlorite and other surfactants like sodium dodecyl sulfate (SDS), betaine, palmitoylcarnitine, and Triton X-100. Sodium hypochlorite is a strong oxidizing agent and is non-selective in nature; these properties can be exploited to disintegrate the non-PHA cellular mass (Yu and Chen 2006). Among other surfactants, SDS showed higher recovery performance. When these chemicals were used individually to extract the PHA, it resulted in a lower yield. However, when a combination of surfactants and sodium hypochlorite are used, the yield of PHA increased (Yu 2009). This method has shown advantages over the solvent extraction and enzymatic digestion because of low cost and easy availability of chemicals. The use of chemicals resulted in a highly crystalline PHA, but they were found to have a reduced purity.

7.5.2.2 Enzymatic Digestion

The method of recovery by using enzymes is a highly complex process. Many enzymes were studied individually and in combinations for extraction of PHA, but the most effective one reported was by using protease. In this method, the organism synthesizing PHA is inactivated by heat after the fermentation period followed by introduction into the culture of protease-producing organism. The protease-producing organism will cleave/lyse the cells and degrade all the cellular content except PHA; this culture can be filtered to obtain PHA easily. The enzymatic method of PHA recovery results in PHA with a very high purity (Lakshman and Ramachandriah Shamala 2006). The same reason makes researchers choose enzymatic digestion although it is complex and costly.

7.5.2.3 Mechanical Disruptions

Mechanical disruption is mainly done for the extraction of cellular proteins with minimal damage (Tamer et al. 1998). The same mechanical disrupting techniques can be used to extract PHA out from the cells. Among different techniques employed, the bead milling and high-pressure homogenization stand out to be highly efficient. In bead milling, small glass beads are homogenized with the fermented media containing cells, and the beads will collide with the cells and transfer the energy at the contact zone which will disrupt the cell. This method is very advantageous as it uses very little energy (Tamer et al. 1998). The high-pressure homogenization technique induces high pressure on the cells which disrupts them. The pressure is regulated by restricted orifice discharge valves (Geciova et al. 2002). The advantages of using mechanical disruption technique are that it does not even interact with the molecules (hence there is no product damage) and minimized environmental pollution and contamination to the products (Kapritchkoff et al. 2006). The main drawbacks of this technique are that it needs high capital investments, takes a very long processing time, and is non-feasible when intended to be used on a large scale.

7.5.2.4 Other Digestion/Disruption Techniques

Other techniques used for PHA recovery are supercritical fluid extraction, cell fragility, flotation, aqueous two-phase system, and gamma irradiation. The use of supercritical fluid for PHA recovery is promising as it possesses high density and low viscosity. Studies are being conducted using different supercritical fluids. The most commonly employed fluid is supercritical carbon dioxide. It has a very low toxicity and reactivity, and the working condition is very suitable (37 °C, 73 atm) and has a minimal effect on PHA molecules. The supercritical carbon dioxide is easily available at a lower cost and is nonflammable, which is an advantage when compared with the normal solvents used for recovery (Hejazi et al. 2003).

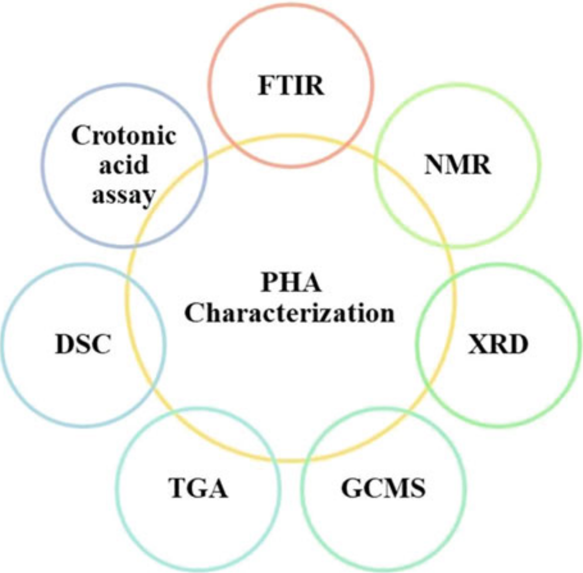
Cell fragility can be attained by adjusting the media compositions. Certain microbes like *Bacillus flexus* when cultivated with a less concentration of certain amino acids and in the absence of diaminopimelic acid increased cell fragility. It was also reported that substances like fish peptone when added to media tend to soften the cell giving large, pleomorphic, and osmotically sensitive cells of *Azotobacter vinelandii*, with high PHA yield (Page and Cornish 1993). By the inducing fragility in cells, we can easily extract PHA, reducing the investments in extraction procedures. Floatation techniques can be used to obtain highly pure PHA with ease. The cells are mixed with solvents like chloroform and then by subjecting to floatation can separate the cell debris and dissolve the PHA which can be recovered easily. The aqueous two-phase system is formed by mixing two polymers (or a polymer and inorganic salt) (which are immiscible) at low concentration (Yang et al. 2008). The two immiscible polymers when mixed tend to coexist, e.g., polyethylene glycol [PEG] 8000/phosphate. When the cell filtrates are treated with these aqueous two-phase systems, the resulting PHA showed high purity (Divyashree et al. 2009). The aqueous two-phase system has a short processing time and minimum cost and consumes very less energy unlike other recovery procedures and gives a good resolution and high yield.

An upcoming procedure for recovery of PHA is by using gamma irradiation. A low gamma irradiation provides optimal cell disruption, and the PHA molecules will be highly pure because no chemicals are used for the disruption (Divyashree and Shamala 2009). Although gamma rays are known to be harmful, data related to gamma irradiation for cell disruptions are not widely available. This method requires more studies to have an assurance to be used as a cell disruption technique for PHA extraction.

7.6 Characterization Methods

Characterization methods play a pivotal role in deducing the molecular architecture of biopolymers. It has a great significance in PHA research as they comprise a whole range of biopolymers with a wide variety of monomeric constituents. The monomeric compositions of PHAs are dependent on the substrate used and the fermentation conditions employed. The monomeric composition of PHAs determines the materialistic properties and application range of these biopolymers. This clearly illustrates the significance of determining the monomeric composition of PHAs (de Rijk et al. 2005). A comprehensive outline of the major characterization methods routinely used for studying PHAs is depicted in Fig. 7.7.

Fig. 7.7 PHA characterization methods



7.6.1 Crotonic Acid Method

This method relies on the conversion of P(3HB) to crotonic acid under an acidic environment provided by concentrated sulfuric acid. The crotonic acid so formed is in proportion to the amount of pH with absorption maxima at 235 nm that could be quantitatively detected through spectrophotometric techniques (Law and Slepecky 1961).

7.6.2 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analysis is used to deduce the functional groups present in the PHAs to differentiate them from other biopolymers. In FTIR analysis, when a specimen is exposed to IR rays, it absorbs energy at frequencies which are characteristic to the particular molecule. The reverse peaks in the IR absorption plot correspond to the specimen's reflectance and transmittance of infrared rays at different frequencies. FTIR is routinely used for rapid detection of PHB and medium-chain-length PHAs (mcl-PHA) (Pandian et al. 2010). These frequencies also depend on the spatial interrelationship of the atoms contained in the molecular unit. Requirement of a minute amount of sample, reliability, accuracy, absence of solvent system, and minimal sample manipulation has made FTIR a routine method for polymer characterization (Oliveira et al. 2007; Priyanka et al. 2020).

7.6.3 Nuclear Magnetic Resonance (NMR) Analysis

Nuclear magnetic resonance (NMR) is a routinely used technique for analyzing the exact location of double bonds in PHA. The monomer could be studied with the aid of 2D homonuclear or heteronuclear techniques. Further, this technique could be used to study all kinds of specialized PHA such as halogenated or acetylated PHA (Williams et al. 1986). NMR relies on the absorption of applied electromagnetic pulse and radiates this energy back. NMR aids in the clear understanding of each functional group present in the polymer with the help of the magnetic resonance. NMR can be used to analyze the monomer composition, metabolic studies, and copolymer analysis (Jacob et al. 1986).

7.6.4 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS remains as the most preferred method for qualitative and quantitative detection of PHA molecules because of its high detection sensitivity and separation power (Korotkova et al. 2002). GC-MS is a highly reproducible and accurate method for reliable detection, quantification, and confirmation of PHA (Braunegg et al. 1978). GC-MS employs chemical ionization and electron impact technique to create characteristic spectra for PHA characterization. Furthermore, tentative identification of novel monomers in the absence of analytical standards can be done through GC-MS (Hahn et al. 1995).

7.6.5 X-Ray Diffraction (XRD) Analysis

XRD is a routine method used to deduce the absolute crystalline structure of polymers. It extends light on bonding pattern of PHA, degradation pattern, and rate of crystallinity. The semi-crystalline and amorphous nature of PHA can be studied from diffractograms using Lorentzian and Gauss functions (Amoozegar et al. 2009).

7.6.6 Differential Scanning Calorimetry (DSC) Analysis

DSC is a routinely used method for studying the melting temperature and glass transition temperature of PHA. It is a thermo-analytical technique that measures energy changes to trace phase transitions. The difference in heat flow between the samples as the reactions may be exothermic and endothermic is measured during DSC analysis (Chen et al. 2009).

7.6.7 Thermogravimetric Analysis (TGA)

TGA is used to measure the thermal stability of the polymer which is a crucial factor in determining its suitability for thermal application. TGA measures the amount and rate of change in the weight of a material as a function of temperature or time in a controlled atmosphere. The differences observed from the thermal degradation are related to the cross-linking isomerization process that is taking place prior to the beginning of the degradation process. The cross-linking process might be affected by the size of alkyl substituent and subsequently the degradation process up on the thermal treatment (Don et al. 2006).

7.7 Challenges in Commercialization and Future Prospects

Despite the continuous efforts and research focus on PHA production, only a very few commercial-scale plants have been developed in the last decades. One of the major challenges in the commercialization of PHA is the high cost of production. The fermentation cost is majorly contributed by substrate cost that accounts for more than 40% of the total production cost (Nath et al. 2008). This issue can be tackled out by considering several factors that have a direct correlation with PHA production like designing of better fermentation strategies, optimization of fermentation process, improving cell's ability to use cheap raw materials for PHA production, and employing recombinant strains for fermentation process (Pan et al. 2012). The second factor that acts as an obstacle for commercialization is the cost associated with the PHA recovery process (López-Abelairas et al. 2015). The accumulation of low concentration of PHAs inside the cell makes it tedious to extract them using any of the extraction process. Even though solvent extraction was found to be an excellent method for PHA recovery from a wide variety of substrates, the concern about the non-eco-friendly nature of such methods still persists. The major areas that need to be focused for commercialization include PHA production from inexpensive raw materials by integrating it with waste management (Koller et al. 2017), designing of eco-friendly extraction process (López-Abelairas et al. 2015), and engineering microorganisms for increased accumulation of PHA.

7.8 Conclusion

The promising potential of PHA as a versatile biopolymer is still not fully exploited due to the issues concerning its production and recovery on a commercial scale. Production of PHA from easily available and cheap raw materials like fruit waste can significantly contribute to the reduction in the overall economics associated with commercial production of PHA. Designing of a sustainable and eco-friendly recovery process without compromising the quantity as well as purity of PHA is also way ahead. Strategic and systematic research on these areas can clearly make these microbial polymers as green alternatives to their synthetic counterparts in the future.

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Bio-plastic Polyhydroxyalkanoate (PHA): Applications in Modern Medicine

8

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Abstract

Bioplastics are in medical use since time immemorial and continuously gaining interest in diverse arenas of science. This includes a class of bioplastic polyhydroxyalkanoates (PHAs) that naturally exhibit a wide range of properties such as thermoplasticity, elastomer behavior, simple tunability, and immunotolerance. Although PHAs possess many advantages, simultaneously it also suffers from fundamental deficiencies such as fragility, low flexibility, and limited surface functionalities. In recent years, several studies have focused on the enhancement of the properties of PHA, particularly for biomedical applications by the incorporation of nanomaterials. The structure and physiochemical

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properties of PHA allow it to blend with various biomaterials and bioactive substances, and many interested composites can be designed based on various biomedical applications. The blended PHA can improve various functionalities such as mechanical properties, hydrophilicity, and time bound degradation under precise physiological environment. Modified PHA perhaps may address many biomedical problems and provide sustainable solution to various problems such as load bearing cartilage, heart chambers, wound grafts, and artificial membranes for kidneys. The enhanced immunotolerance, sustained biodegradability, and low toxicity of PHA fascinated various researchers, including tissue engineering, which have demonstrated many medical advances like bioabsorbable sutures (FDA approved 2007), 3D structures, and various medical devices. In future, intelligent auto controllable PHA products like microsphere may perhaps take more responsibilities rather than just a drug delivery system. This chapter provides a comprehensive study of cutting-edge approaches to biomedical applications and potential future strategies.

Keywords

Bio-plastic · Polyhydroxyalkanoate · Biodegradable · Biocompatible · Nanocomposite · Biomedical applications

8.1 Introduction

Modern science has brought many breakthroughs in polymer and biopolymer research globally. Biopolymers encompass many macromolecules, which are biocompatible, noncarcinogenic, biodegradable, and easily tunable. Among other polymers, polyhydroxyalkanoates (PHA) are a group of natural polyesters, generated by microorganisms as energy compound under excess carbon source and restrictive nutrient conditions. Beijerinck in the year 1888 observed macrogranules (PHA) in bacterial cells, and Macrae et al. for the first time concluded that PHA is a reserve material in 1958 (Macrae and Wilkinson 1958). The composition of P(3HB) granule (*Bacillus Megaterium*) is phospholipids 0.46%, proteins 1.87%, and 97.7% polyester, which is proposed by Griebel et al. (1968). Later in the mid-twentieth century PHA gained much scientific attention. Ever since, PHA is continuously investigated in various fields mainly in modern medicine, tissue engineering scaffolds, antibacterial biomaterials, and drug carrier agents (Li et al. 2016). Polyhydroxyalkanoate has been an extensively studied biopolymer as revealed by the published research articles in the last 20 years as shown in Fig. 8.1a, and the worldwide distribution of PHA in biomedical use has been demonstrated in Fig. 8.1b, data obtained from Google Scholar. PHAs are bio-harmonious simple macrocompounds formed by numerous broad-spectrum microorganisms under unfavorable growth condition (Fig. 8.2) (Lee 1996a; Sudesh et al. 2000). In the normal cell growth condition, 3-ketothiolase enzyme is inhibited by coenzyme A, which leaves Krebs cycle. During unfavorable circumstances, depletion of essential nutrients (phosphate, nitrogen, and oxygen) may produce higher amounts of acetyl-CoA instead of CoA within the cell. This flinches the

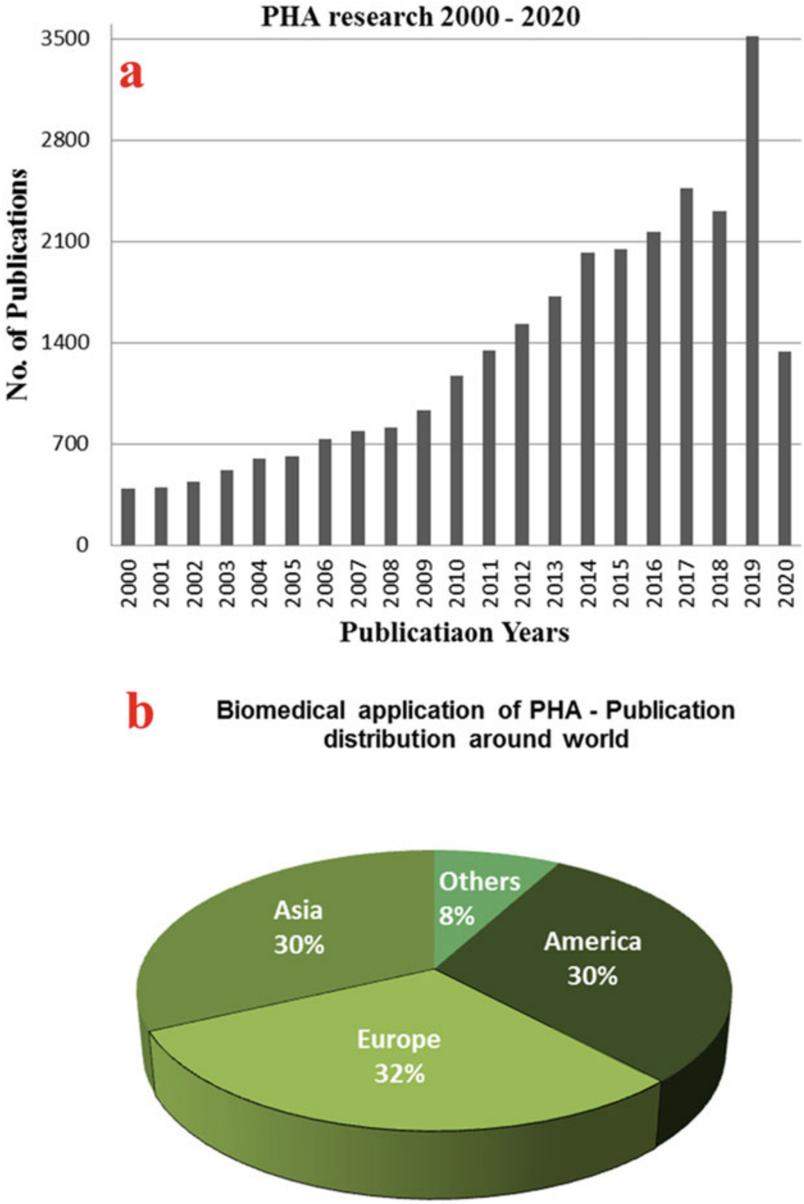


Fig. 8.1 (**a**) Scientific publications in the last 20 years for the term "Polyhydroxyalkanoate"; (**b**) illustrates worldwide distribution of publications on "PHA biomedical applications" (Mukheem 2019)

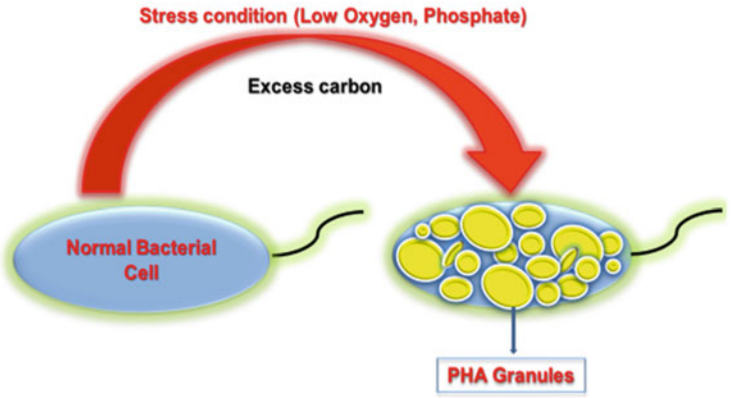


Fig. 8.2 Bacterial cell preserves energy granules (PHA) in an unfavorable condition (Mukheem 2019)

synthesis of Poly 3-hydroxybutyrate P(3HB), through the mechanism of condensing two acetyl-coenzyme A molecules. The refined PHA could be stored or used in various biomedical applications due to its various properties such as biocompatible nature, biodegradability, porosity, flexible structure, thermoplasticity, optically active, and piezoelectric (Reusch et al. 1992; Chen 2009; Chen and Wu 2005; Dhandayuthapani et al. 2011; Grage et al. 2009). The PHA degradation metabolites are nontoxic and are eliminated by natural metabolic processes of body (Kehail et al. 2017). Presently, P(3HB) has gained copious attention in various fields and is also expected to be very useful in numerous biomedical applications (Hocking et al. 1995; Li et al. 2016). The PHA polyesters are composed of many hydroxyalkanoic acids (saturated and unsaturated), which possess many interesting mechanical, biological, and tunable properties. Furthermore, the properties of PHA may be significantly enhanced by blending with suitable materials to achieve the required medical application. Amazingly, PHA is a wholly biocompatible polymer with no carcinogenic effect (Peng et al. 2011). Additionally, PHA is nonpyrogenic, nonirritant, nontoxic, nongenotoxic, and nonhemolytic (Misra et al. 2006; Odermatt et al. 2012) and absolutely degradable at embedded site in the body via natural metabolic pathways (Ozdil and Aydin 2014; Philip et al. 2007; Luckachan and Pillai 2011; Tokiwa et al. 2009).

PHA investigation and its outcome have gained much superiority in the medical field. In the year 2007, the Food and Drug Administration (FDA) has approved TephaFLEX® suture fabricated using poly 4-hydroxybutyrate P(4HB). The biomaterials of P(4HB) have demonstrated better features as compared to polydioxanone (PDSII®) and polypropylene (Prolene®) (Williams et al. 2013). Recently, PHA is widely applied in tissue engineering, regenerative medicine (Chen and Wu 2005; Li et al. 2008; Masaeli et al. 2013), wound management (Gumel et al. 2015; Pramanik et al. 2015), and in drug delivery systems (Errico et al. 2009; Yao et al. 2008). Though the PHA has many unique properties, much remains to be achieved, such as engineering, synthetic pathways to develop improved quality of PHA granules in a limited time period, functional group modification, and minimal to zero cost extraction with no toxicity. Considering the prominence of PHA, this chapter highlights the enriched characteristics of PHA in particular on biomedical applications, limitations, and upcoming prospects.

8.2 Synthesis of PHA

More than 300 microorganisms, including *Cupriavidus necator* (*C. necator*), *Cyanobacteria*, *Pseudomonas putida*, and *Escherichia coli*, naturally produce the PHA granules (Steinbüchel et al. 1992; Hocking et al. 1995; Akiyama et al. 2003; Kaneko et al. 2006). In a nutrient shortage condition, enzyme citrate synthase is inhibited; thereby an elevated number of acetyl coenzyme A is generated instead of CoA, inside the cell. Henceforth, the access of acetyl CoA is restricted into the citric acid (Krebs) cycle and the process of PHA synthesis investigated. As yet, eight biosynthetic cycles of PHA are in place; among which three key cycles are elucidated (Fig. 8.3) (Masood et al. 2015; Chen 2010). *C. necator* is the most investigated bacteria, which contain three core enzymes such as β-ketothiolase,

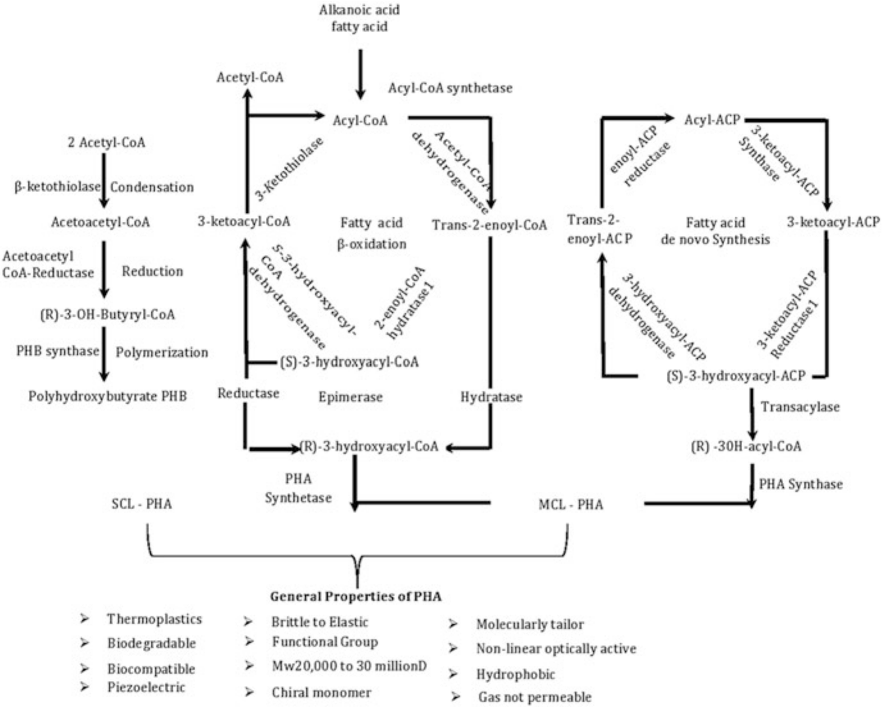


Fig. 8.3 Sketch illustrating biosynthetic cycle of PHA and general properties of PHA (Mukheem 2019). (*CoA* coenzyme A, *SCL* small chain length, *MCL* medium chain length, *ACP* acyl carrier protein)

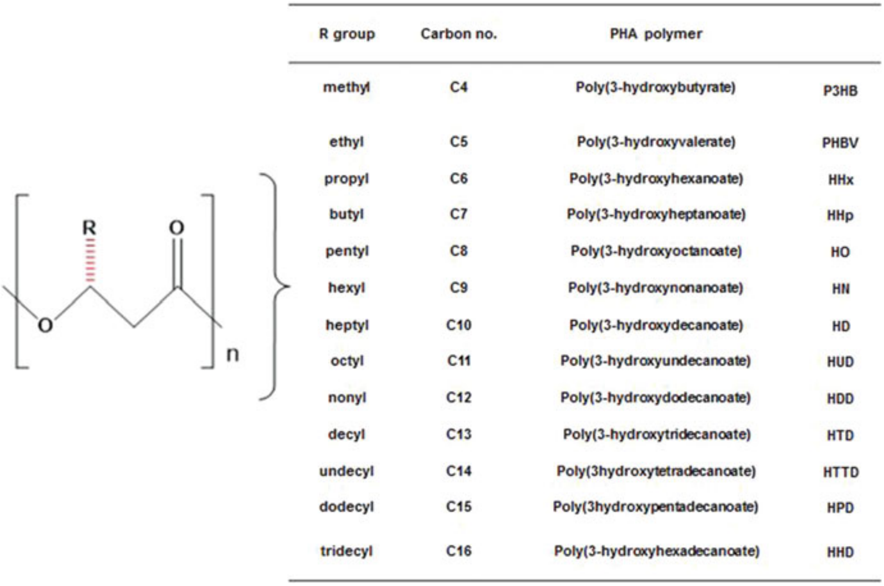
reductase (NADPH-dependent acetoacetyl-CoA), and synthase (PHA) in the production of PHA granules. At the commercial production, the selected strains of *C. necator* is used to produce different monomers of PHA such as poly-(R)-3-hydroxybutyrate-*co*-(R)-3-hydroxyvalerate (PHBV), poly-(R)-3-hydroxybutyrate-*co*-4-hydroxybutyrate (P(3HB-*co*-4HB)), and poly 3-hydroxybutyrate P(3HB). Based on the carbon source provided to microbe, the –R group has the ability to change into diverse types of PHA monomers. Though the findings are in place, PHA bulk production and biomedical applications are still at the academic level. To achieve bulk production many efforts are required, such as selection of potential host and modification of PHA synthesis pathways. The environmental problems, petroleum reserves reduction, and lethal gases linked with synthetic plastic could be addressed through large-scale manufacturing of PHA. For a maximum accumulation of PHA, cell's molecular rejuvenation is required. Additionally, precise augmentation of fermentation conditions may inhibit the formation of energy granules (PHA) at premature log phase. This step aids exploiting the mitosis phase to develop an excessive quantity of cells that feasibly results in a higher PHA yield. Besides, it is essential to emphasize on the extraction protocol to obtain higher quantity of PHAs.

8.3 Types of PHA

In monomeric component of PHA the sum of carbon atoms at side chain broadly classified PHA into three types as shown in Table 8.1 (Mukheem 2019). They are long chain length (LCL) PHA that have >14 carbon atoms at lengthy fatty acid, 6–14 carbon atoms are there in medium chain length (MCL) PHA, and a short chain length (SCL) PHA contains 3–5 carbon atoms (Lee and Chang 1995; Kunasundari and Sudesh 2011). The side chain of PHA molecule contains the functional groups including fatty acids. Figure 8.4 demonstrates the various types of PHA based on their carbon atoms (Shrivastav et al. 2013; Tan et al. 2014). Approximately, there are 150 different monomeric components found in homo- or copolymers of PHA which possess a range of properties (Sudesh et al. 2000; Steinbüchel and Lütke-Eversloh 2003). The microorganism synthesizes a range of PHA through the influence of a

Table 8.1 Types of PHA

Bacteria	No. of carbon atoms	Polymer type	Types of PHA	References
Ralstonia eutropha	C3–C5	P(3HB), P(4HB)	Short chain length	Yan et al. (2005), Rai and Roy (2011), Green et al. (2002)
Recombinant *E.coli*	C6–C14	P3HHX, P3HO	Medium chain length	Rai and Roy (2011), Klinke et al. (1999)
Pseudomonas aeruginosa MTCC 7925	C15–C18	P(3HB)-*co*-3HHX	Long chain length	Singh and Mallick (2008)



R group	Carbon no.	PHA polymer	
methyl	C4	Poly(3-hydroxybutyrate)	P3HB
ethyl	C5	Poly(3-hydroxyvalerate)	PHBV
propyl	C6	Poly(3-hydroxyhexanoate)	HHx
butyl	C7	Poly(3-hydroxyheptanoate)	HHp
pentyl	C8	Poly(3-hydroxyoctanoate)	HO
hexyl	C9	Poly(3-hydroxynonanoate)	HN
heptyl	C10	Poly(3-hydroxydecanoate)	HD
octyl	C11	Poly(3-hydroxyundecanoate)	HUD
nonyl	C12	Poly(3-hydroxydodecanoate)	HDD
decyl	C13	Poly(3-hydroxytridecanoate)	HTD
undecyl	C14	Poly(3hydroxytetradecanoate)	HTTD
dodecyl	C15	Poly(3hydroxypentadecanoate)	HPD
tridecyl	C16	Poly(3-hydroxyhexadecanoate)	HHD

Fig. 8.4 R group with carbon numbers and types of PHA co-polymers (Mukheem 2019)

polymerizing enzyme PHA synthase (PhaC) (Jendrossek 2009; Bhubalan et al. 2011). PHA-producing microbe uses various carbons and substances to yield choice of PHA granules (Madison and Huisman 1999), such as poly-3-hydroxybutyrate, poly-3-hydroxybutyrate-*co*-3-hydroxyhexanoates (PHBHHx), poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (PHBV), and poly-3-hydroxyoctanoate (PHO) (Pandian et al. 2009; Yu and Stahl 2008; Lorrungruang et al. 2006; Sankhla et al. 2010; Ouyang et al. 2007; Qin et al. 2007).

8.4 Properties of PHA

PHA homopolymers consist of 3–5 carbon molecules, which demonstrate the characteristics of polymer brittleness and stiffness. Many PHA monomers have been found such as hetero-, homo- and copolymers depending on their chemical configuration and side chain length. The properties of PHA monomer are similar to that of isotactic polypropylene (petroleum) such as flexibility, thermal, and mechanical. The biodegradability property of PHA is depicted in Table 8.2 (He et al. 1999). Microbial P(3HB) has several physiognomies such as Young's modulus (3.5 GPa), elongation break (10%), tensile strength (43 MPa), melting temperature (180 °C), semicrystallinity (1.26 g/cm^{-3}), glass transition temperature (5 °C), molecular mass range ($1 \times 104^{-3} \times 106$ g/mol), polydispersity (2), and amorphous (1.18 g/cm^{-3}) (Brigham et al. 2011; Budde et al. 2011; Sudesh et al. 2000; Holmes 1988; Barham et al. 1984). The elastomers or rubbers have closely resembled MCL-PHA, which possesses low melting and low glass transition temperature. For injection molding,

Table 8.2 A summary of biomedical studies for PHA biodegradability

PHA	Biomedical applications	Conclusion	References
β-hydroxybutyric acid P(3HB)	Microsphere implants	Continuous drug delivery for longer time period	Shishatskaya et al. (2008)
P(3HB), PHBV P(3HB-4HB) composite	Implants for treatment of osteomyelitis An antibiotic dissolution rate (polymer degradation assessment)	Sustained release of antibiotics for 2 weeks at a continuous rate	Türesin et al. (2001)
PHB/atactic		Post-implantation (26 weeks), residuals found in a single animal among four experimental animals	Freier et al. (2002)
P(3HB), P(HB-*co*-HHx)	The optimized film P(HB-*co*-12%-HHx) has decomposed faster, as compared to other films	Amorphous and irregular topology decomposes fast in activated sludge	Wang et al. (2004)
PHBHHx	PHBHHx films treated with ultraviolet, conserved 92 and 87% mass wrt their original weight	Ultraviolet energy imposed for controlled decomposition	Shishatskaya et al. (2008), Wei et al. (2009), Ji et al. (2009), Shangguan et al. (2006)

lower molecular weight and comonomers of PHA are appropriate. The medium molecular weight PHA consists of 10% comonomers that are suitable for film casting, thermoforming, and laminations. For gloves, the higher molecular weight PHA containing 15% comonomer (softer, adhesive, and elastics) is suitable (Bugnicourt et al. 2014). The aforementioned characteristics made PHA to be applied in numerous fields including biomedical applications (Valappil et al. 2006; Li et al. 2016), packaging industries (Shen et al. 2009), and drug delivery (Sendil et al. 1999).

8.4.1 Biodegradability and Biocompatibility

The decomposing of PHA may occur via biological, chemical, or physical processes. Biodegradation of PHA occurs through lipase, esterase, and depolymerize enzymes (Mukai et al. 1993; Doi et al. 1990), which hydrolysis PHB into hydroxyacids (oligomers and monomers). Generally, the biodegradation and resorption of PHA takes between 12 and 18 months (*in vivo*) with no toxic residuals (Tokiwa et al. 2009; Philip et al. 2007). However, it varies with size and type of processing (Williams et al. 2013). P(4HB) film in the physiological conditions can degrade into 4HB, H_2O, and carbon dioxide excreted from body. In mammals, 4HB element is typical found in kidneys, heart, liver, lung, brain, muscle, and adipocytes (Nelson et al. 1981). Basically the degree of PHA biodegradation is associated with various environmental factors such as shape, size, crystallinity, porosity, surface area, hydrophobic/hydrophilic nature, chemical composition, molecular mass, including pH, temperature, moisture, species, and nutrient supplies (Boopathy 2000; Lee 1996b; Shishatskaya and Volova 2004; Sodian et al. 2000b). In a study, the PHB films implanted subcutaneously in Wistar rats have shown different degradation patterns based on film thickness (50 mkm) and Mw (1000 kDa) (Bonartsev et al. 2007). At various time points (0.5, 1, 2, 3, and 6 months) the films are isolated and examined for degree of crystallinity, weight, and Mw. Initial time interval has revealed 2% degradation followed by 6% in 2 months, 72% and 100% in 2.5 and 3 months, respectively (Bonartsev et al. 2007). Chronology of biodegradation of PHB embedded scaffolds can be understood that first it gets enclosed in a fibrous capsule that increases its crystallinity and decreases its Mw. These changes are collectively responsible for the major breaks including fragmentation that completes the degradation (Bonartsev et al. 2007; Qu et al. 2006b). Freier et al. (2002) has demonstrated the degradation of PHB film dipped into the sorensen buffer at the following conditions: pH 7.4 and 37 °C temperature for 1 year has reduced to one-half from 5 mg weight (Freier et al. 2002). Similar PHA degradation studies have been described in Table 8.2. To further improve the PHA abilities, the modified PHA scaffolds may accomplish sustained drug delivery, intended quantity of drug and active molecules delivery at various time points. Biodegradation is one of the exceptional characteristics in various applications of PHA besides biocompatibility.

A biocompatible element for medical applications should not be cytotoxic and noninvasive in the biosystem. Numerous studies proposed PHA polymer as biocompatible at the grafted site in a biological environment (Table 8.3). PHA molecule migration may associate with tissue response. For example, a biomaterial is supportive for subcutaneous tissue, but may not be compatible for peritoneum or nerve tissue (Kohane et al. 2006). Odermatt (Odermatt et al. 2012) described a P(4HB) implant that has shown excellent biocompatible properties (no irritation, toxicity, pyrogenicity, genotoxicity, and chronic toxicity) intramuscularly and completely absorbed in 64 weeks at the grafted site (Odermatt et al. 2012). However, negligible tissue response and tenderness were recorded, parallel to FDA approved silk biomaterial (Altman et al. 2003). The primary inflammation of PHA implant is perhaps associated with trauma such as injection or surgery, which may disappear by time (Amass et al. 1998). Few polymers (silicone–polysiloxanes) are suspected to provoke cancer (Cammas et al. 1999). Hence, biocompatible PHA is safe and useful for emerging numerous medical applications such as cardiovascular, orthopedic, nerve guides, drug delivery vector, wound management, tissue engineering, and regenerative medicine (Chen and Wu 2005; Valappil et al. 2006; Hazer 2010). The aforementioned properties of PHA can be enhanced by PHA modification that may produce biosmart composite, which perhaps attain several responsibilities by auto sensing the various conditions of human physiology.

Table 8.3 PHA and their medical applications

PHA	Host or scaffold	Applications	References
P(3HB)	Rat, sheep, rabbit, cat, human	Nerve repair, pericardial patch, hard tissue scaffolds	Volova et al. (2003), Novikov et al. (2002), Mosahebi et al. (2002), Unverdorben et al. (2002), Löbler et al. (2002), Hazari et al. (1999), Malm et al. (1992), Malm et al. (1994)
P(4HB)	Sheep, rat	Cardiovascular valves, nerve guides	Opitz et al. (2004), Stock et al. (2000b), Hoerstrup et al. (2000), Bian et al. (2009)
P(3HB-*co*-3HV	Rat, dog, rabbit	Urological stent, hard tissue engineering	Volova et al. (2003), Leenstra et al. (1998), Kostopoulos and Karring (1994)
P(3HB-*co*-3HHx)	Dog	Artificial esophagus	Chen and Wu (2005)
P(3HHx-*co*-3HO)	Sheep, rat	Cardiovascular grafts	Stock et al. (2000a), Shum-Tim et al. (1999)
P(3HB), P (HB-*co*-HV-*co*-HHx)	PHA-based scaffolds	Adherence and proliferation of stem cells on PHA membranes	Shishatskaya et al. (2008), Wei et al. (2009), Ji et al. (2009)
PHB, PHBV, and PHUE-O3	PHA biofilms	Fibroblast cells attachment (85–99%)	Rathbone et al. (2010)
(PHBHHx/PHB), PHB	3D scaffolds with permeability	Chondrocytes proliferated and well maintained its phenotype for 28 days	Deng et al. (2002)
P(4HB)	N/A	Hernia repair	Martin et al. (2013)

8.5 Applications in Tissue Engineering and Regenerative Medicine

The fundamental concept of tissue engineering and reformative biology is that biopolymers should imitate several duties of the extracellular matrix and target tissues which are proposed to be repaired. Biopolymers are anticipated to encourage the auto repair process of the host. Additionally, it ought to deliver mechanical support to damaged tissue at the remodeling phase, leaving nontoxic fragments in the host.

8.5.1 Orthopedic

PHA composites have been used to treat the hard tissue (load-bearing) applications, which is a unique approach of hard tissue engineering. The great challenge of orthopedic is the lack of donors and limited self-repair ability of the host, which could be addressed moderately through the suitable nanocomposites. An example of a good orthopedic scaffold feasibly should guide cell migration, attachment and proliferation and excite differentiation of stromal cells until maturity phase. Poor guidance of differentiated cells (osteocyte or chondrocyte) may dedifferentiate and lose particular phenotype which may lead to hypertrophy (cell death mechanism, morphologically different from apoptosis) or osteoblast. The crystalline nature, flexibility, and decomposing profile of PHA certainly SCL and MCL polymers are ideally useful in hard tissue engineering (Misra et al. 2006). MCL-PHA contains low tensile strength that may improve load-bearing ability of composites. In PHA family, P(4HB), hydroxyhexanoate, hydroxyoctanoate, and hydroxyvalerate are apt materials for hard tissue applications. In a study, P(3HB) copolymerization enhanced the mechanical characteristics alongside better adhesion, improved proliferation and differentiation (Chen and Wu 2005; Kitamura and Abe 1995). Similarly in a study, PHB demonstrated a rapid bone formation at implanted material with consistent bone tissue adaptation with minimal inflammation for a year (Doyle et al. 1991). Mesenchymal stem cells isolated using human bone marrow were seeded on a composite of PHBHHx/arginyl-glycyl-aspartic acid (Phap-RGd), which produced ample ECM, sulfated glycosaminoglycans (sGAG), collagen (type II), SOX-9, and aggrecan (You et al. 2011). The homogeneous growth of hBMSCs is seen on PHBHHx-Phap-RGd scaffold as compared to Phap-coated biomaterial after 4 and 72 h. This result suggests PHBHHx-Phap-RGd-fabricated scaffold is appropriate for chondrocyte formation and hard tissue engineering (You et al. 2011). Rabbit chondrocytes were embedded on 3D PHBHHx scaffold, implanted in rabbit, and assessed after 4 months. The result demonstrates a progressive cartilage formation, such as adequate thickness and better ECM distribution with improved surface integrity, compared to control (Wang et al. 2008). A study demonstrated the blend of PHB:PHBHHx (1:1) scaffold which has better mechanical properties such as improved surface energy and ideal adhesion material for chondrocytes. PHB: PHBHHx scaffold has revealed greater (five times) adhesion ability after 24 h as compared to PHB films, which possess week adhesion of chondrocytes (Zheng et al. 2005). In another investigation, PHB:PHBHHx (1:2) scaffold has shown excellent GAG formations and increased collagen content to fourfold over time as compared to PHB alone. The result suggests the fabricated fraction of composite perhaps greatly supportive for ECM generation to guide articular cartilage (Deng et al. 2003). PHB-bioactive glass (45S5) composite foams seemly improve the osteoblast cell (MG-63 strain) adhesion and growth in rats with no immune reaction even after a week of implantation (Misra et al. 2010). Owing to the promising features, the PHA polymer continuously gains much attention to develop composites and devices for hard tissue engineering (Misra et al. 2006; Zinn et al. 2001). Additional insight into PHA-based suitable design scaffold is required to channel up chondrocyte differentiation, and governing chondrocyte is mandatory to stop dedifferentiation or retransformation including hypertrophy.

8.5.2 Cardiovascular

Throughout the world the main risk of death is heart disease among other major threats and has been increasing over time (Kawano and Arora 2009). It is essential to investigate the factors responsible for faulty veins and arteries and to develop a potential cardiovascular composite for its repair mechanism. In the current cardio applications, most of the implants (allograft, xenograft, artificial graft, and autologous tissue) are linked with many disadvantages such as thromboembolism, endocarditis, calcification, immune response, prosthetic valve, and poor stability (Kurobe et al. 2014). Therefore, an ideal biocomposite should have enormous strength, precise flexibility, and durability to continue the mechanical cycles of blood loading with every heartbeat. Several investigations reported that the PHA composites possess stiffness and elastomeric capacity to bear cyclic stress and are noninvasive implants (Generali et al. 2014).

Fabricated trileaflet heart valve composite possesses a cylindrical stent size of 1 mm × 15 mm × 20 mm with a three-leaflet valve, which is further modified to form pores (80–200μm size) by salt leaching technique. The composite was embedded with vascular cells followed by incubation, and after 4 days the movements of leaflet were recorded through pulsatile flow bioreactor (Sodian et al. 2000c). A two centimeter slice of pulmonary artery was employed using autologous pre-seeded scaffold, in place of the native pulmonary leaflets valve. During the observational period all experimental animals survived; the group is sacrificed at 1, 5, 13, and 17 week intervals respectively. The resulting constructs were covered predominantly by GAG, ECM, collagen, tissue, and nonthrombus. The result of the aforementioned study suggests that the PHA based composites may be potential for pulmonary implants with essential duties (Sodian et al. 2000a). In a study, preseeded polyglycolic acid-P4HB scaffold is implanted in the lamb abdominal aorta, which over time mimics as the native blood vessels (Shum-Tim et al. 1999). After 4 weeks of culturing, the polyglycolic acid-P4HB heart valves (trileaflet stented) were localized. Additionally, the dynamic and static conditions were observed with dense GAG and collagen content in regular tissue as compared to control groups. Polyglycolic acid-P4HB impacts growth and remodeling is the primary evidence of living pulmonary arteries. This scaffold enriched with growth factors and ascorbic acid has demonstrated good deposition of collagen and human pediatric aortic cell proliferation (Mol et al. 2006; Fu et al. 2004). Thereby, engineering PHA with suitable composites could be a more useful biocomposite for cardiovascular applications (Chen and Wu 2005; Chen et al. 2001; Dai et al. 2009).

8.5.3 Nerve

Nerve construction is a comprehensive biological cascade. Generally, nerve consists of sensory, motor, and sympathetic components. In the peripheral nervous system (PNS) slight damages are reformed through self-healing phenomenon and irreparable damages must go through the nerve autograft treatment. However, the auto-repair mechanism for neurodegenerative and PNS disease, particularly spinal cord restoration, is impeded by a few factors. But nerve tissue engineering with recent advances in neuroscience has provided optimism for nerve repair applications. Nerve guide composites should ideally carry multiple tasks in repair mechanism. PHA base scaffolds are widely used to attain many neuroscience approaches. A study demonstrated that the composite of PHBHHx had shown extraordinary support to neurocyte, finally forming the neurons, whereas P(3HB) and P(4HB) have shown low performance (Xu et al. 2010). PHA mats (2D and 3D) are premeditated through phase separation method to mimic as ECM. The outcomes revealed that 3D matrices have shown better attachment, proliferation, and neurite formation, which is potentially a realistic mat for CNS damage repair (Xu et al. 2010). In a similar study, PHB/VHHx 3D scaffold has promoted hBMSCs differentiation to nerve cells, whereas 2D scaffold has shown no better results. Moreover, hBMS cells have shown enhanced nerve cell differentiation with the scaffold pore size ranging from 30 to 60μm. Besides, cell attachment and proliferation results were confirmed through quantifying β-III tubulin and nestin nerve markers (Wang et al. 2010).

Bian et al. (2009) reported the biocompatibility of PHBHHx nerve conduits with consistent porosity and nonconsistent porosity of walls. The conduits are implanted at sciatic nerve to channel a ten millimeter defect of adult rat (Sprague-Dawley). The results revealed that both the conduits are permeable to the free exchange of nutrients, including glucose, bovine serum albumin, and lysosome. The mechanical properties of nerve conduits have shown optimum stress of 2.3, 0.94 MPa and finest load of 3.1, 1.3 N for consistent and nonconsistent walls respectively. After a month, mechanical properties of implanted conduits revealed improved functional recovery of damaged nerves by complex muscle action in SD rats (Bian et al. 2009). PHBHHx composite has good crystallinity as compared to PHBHHx/PDLLA composite. From the result, it is depicted that the exceedingly aligned micro-architecture possesses the essentials of native nerve tissue and therefore suitable for long nerve gap applications (Gao et al. 2006). The fabricated PHBV/collagen (ratio 50:50) composite has shown higher nerve cell proliferation (PC12); other composites have shown weaker cell growth (Prabhakaran et al. 2013).

8.5.4 Drug Delivery

Drug delivery is a multidisciplinary developing arena of modern science, which has been widely studied to improve the appropriate application's ability. The current drug delivery system has been suffering from a few limitations such as less influence on the target site, high cytotoxicity, suboptimal bioavailability, and unsuitable volume of drug release. The effective drug delivery practices are essential and can be achievable by insight into the controlled drug delivery system and release of pharmacological substances to particular cells, tissues, and organs. In recent times, advances in biomedical engineering have been offering effective vectors and delivery system such as microspheres, tiny molecule drugs, proteins, oligodeoxynucleotides, peptides, plasmid DNA, highly biocompatible and degradable biomaterials. Biopolymers perform dynamic share in sustainable and continuous drug delivery system by assisting fabrication of suitable vectors. PHA-associated physiochemical and mechanical properties and its natural occurrence are considered to be useful among other drug delivery candidates (Mukheem et al. 2019). Lu et al. (2011) reported that PHA nanoparticles have proven as better transporter of TGX221, an inhibitor of PI3K in anticancer investigation. The continuous release of antibiotics, nanoparticles, vaccines, anticancer and anti-inflammatory agents, anesthetics, steroids, and hormones is achieved using PHA microspheres, microcapsules, and scaffolds (Nobes et al. 1998; Orts et al. 2008; Mukheem et al. 2020).

In a study, PHA embedded with graphene-silver nanocomposite has demonstrated significant release of nanoparticles into the culture broth of multidrug-resistant model organisms, which revealed improved destruction of bacterial cells as compared to PHA alone (Mukheem et al. 2018). The tiny microspheres with high permeability are suggested to carry a high amount of drugs. The PHB homo-, copolymers and suitable blends are considered as effective controlled release vectors of drug delivery system. However, copolymers have demonstrated harmonic porosity, which tone down spurt drug release at aimed point; therefore it is beneficial for designing continuous delivery of healing bionanomaterials (Kim et al. 2000; Rossi et al. 2004; Gangrade and Price 1991). In a study, PHB and PHBHHx nanoparticles ranging from 150 to 250 nm have efficiently entered the macrophages without damaging the cell. The fabricated nanoparticles sustainably release the gentamicin and 5-fluorouracil drugs continuously for more than 20 days (Francis et al. 2011; Lu et al. 2010). An ample amount of PCL in PHBV/PCL microparticles demonstrated the improved pore size and therefore augmented the release rate of loaded drugs (dexamethasone, indomethacin, diclofenac). Moreover, diclofenac and indomethacin have liberated significantly faster than dexamethasone from the PHBV/PCL microparticles (Lionzo et al. 2007; Poletto et al. 2007). In a study, the limiting factor of PHA (hydrophobic nature) is modified using hydrophilic block copolymers with 1:1 ratio, which improved ibuprofen release as compared to 3:1 ratio blend and PHB alone (Bidone et al. 2009). PHA nanoparticles loaded with TGX221 (PI3K inhibitor) significantly stop the growth of cancer cell line. Dealing with an oral drug delivery system of colon cancer, the drug must release at the colon (alkaline condition) passing through the highly acidic environs. To accomplish this challenge Chaturvedi et al. (2011) developed PHB/ionic cellulose acetate phthalate (CAP) composite, a pH responsive microsphere loaded with anticancer drug (5-fluorouracil) shown in Fig. 8.5(1). The results revealed that fabricated PHB/CAP microsphere has released 12% drug at 1.2 pH (acidic) in 2 h of time as compared to PHB microsphere released 20% drug. A significant release of 72% drug was noted from PHB/CAP microsphere at 7.4 pH (alkaline) within 24 h. The SEM images

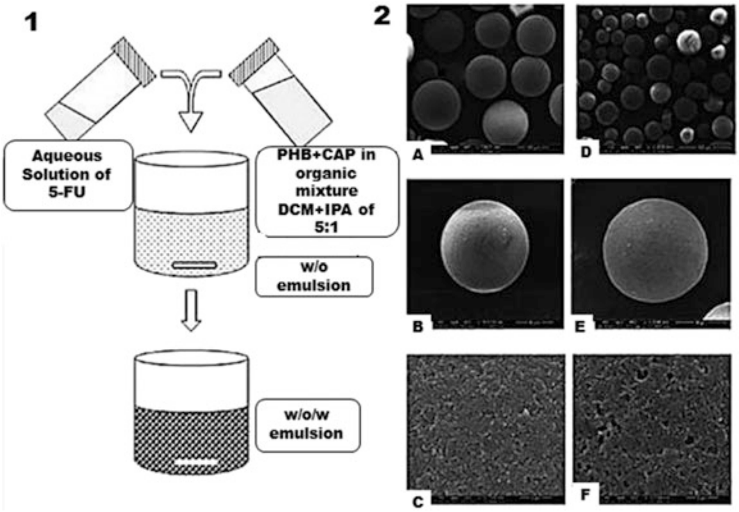


Fig. 8.5 (1) pH-responsive microspheres preparation; (2) dissolution, SEM images of microsphere at various magnifications ×250 (*a*), ×600 (*b*), ×5000 (*c*), ×140 (*d*), ×800 (*e*), and ×5000 (*f*). SEM image *a–c* represents 5-FU/PHB microsphere and image *d–f* signifies 5-FU-loaded PHB/CAP composite (Chaturvedi et al. 2011)

demonstrate the fine pores develop in microsphere by dissolving CAP in alkaline condition, leaving PHB alone with fine pores that releases a profuse amount of drug as compared to an acidic condition shown in Fig. 8.5(2F), whereas PHB microsphere has remained undissolved in acid and alkaline conditions (Fig. 8.5(2)) (Chaturvedi et al. 2011).

8.5.5 Wound Management

Many PHA products contributed to wound management, to name some sutures, surgical mesh, biodecomposing mats, and skin regeneration scaffolds (Bonartsev et al. 2007; Chang et al. 2014). The conventional fibrous mats (gauze, greasy gauze) (Jones 2006; Hoekstra et al. 2002) are used for wound management but are not suitable due to disruption of wound bed and scar formation (Kuppan et al. 2011). Many studies demonstrated PHA as a suitable material for wound management due to its adaptable biological, mechanical, and degradable properties including drug delivery in time at physiological environments. Zonari et al. (2015) stated that fibroblastic cells of human skin are attached and grow significantly on PHBV 2D and PHBV mats. The result revealed a significant increase of elastin and collagen markers and weaker expression depicted in control and tissue culture polystyrene. The result suggests that PHBV is a potential scaffold, which accelerates re-epithelization by stimulating collagen production (Zonari et al. 2015). In a similar investigation, PHBV biomaterial embedded with adipocytes has stimulated bFGF and VEGF gene expression. This aids the development of proper blood vessels and

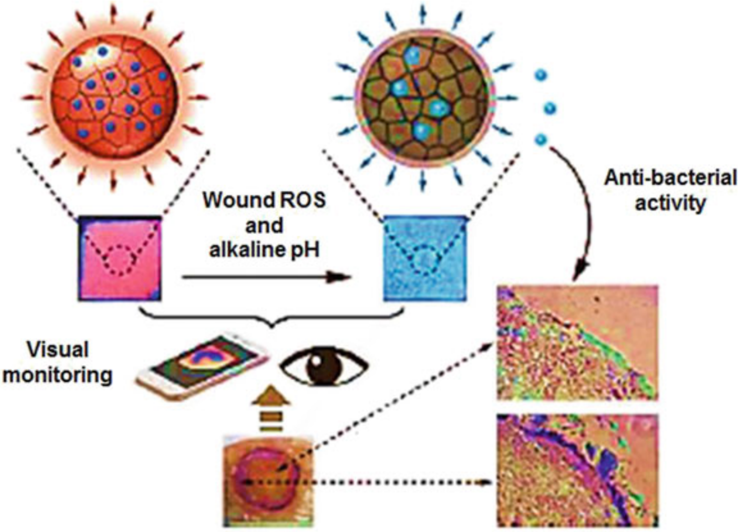


Fig. 8.6 A dual luminescent smart bandage system for wound management (Chen et al. 2017)

regulates α-SMA, TGF-β1, and TGF-β3 for scars, which are the key factors of wound management (Leenstra et al. 1998). Additionally, a study demonstrated that PHA-associated materials have the suitable Young modulus, tensile strength of 15–150 and 5–30 MPa respectively, which are equivalent to human skin (Li et al. 2008; Panaitescu et al. 2017). Therefore, the aforementioned PHA-associated properties are mechanical, functional, and biologically fit for wound management.

Several routine therapies in wound management are required to change the applied wound membrane frequently that perhaps damages the newly formed wound cover. In forthcoming years, the frequent replacement of wound scaffold in short time duration can minimize by a few times to zero through responsive system. In a study, a smart scaffold has been designed that continuously monitors and facilitates wound management. The binary luminous system changes the pigments of scaffold from red to blue in response against high pH by sensing reactive oxygen species (Fig. 8.6). Similarly, it is suggested that fluorescent dyes can be employed to specify drug availability in the embedded scaffold through sensing and transferring colors from red to orange and gradually moving to yellow (100 to 20%). The yellow color can be used as an alarm indicator for biomaterial replacement as early as possible, and the colorless mat signifies nearly 0% drug availability. So far, there is no such investigation in place, to the best of our knowledge, on the monitoring of drug availability alarm in current wound management applications. This type of smart materials could seriously protect the enormous wastage of drug due to frequent change of the wound bed. Furthermore, it also minimizes the disturbance of newly formed wound foundation and the precious time of health professionals.

In a similar study, flexible bioelectronic bandage fabricated using stretchy polymer has demonstrated a well-sensing and drug release system (Farooqui and Shamim 2016). In this aspect, the MCL-PHA (elastic/rubber) are more suitable candidates for ongoing wound management advances. The flexible bioelectronics smart bandage allows insight into the ongoing real scenario of the wound healing cascade by signaling the true information. Additionally, the smart bio-mats are adjusted through many related factors, oxygen, pH, moisture, temperature, and pressure including infection load and auto release mechanism (McLister et al. 2016; Mehmood et al. 2014). Kassal et al. (2015) (Fig. 8.7) fabricated a smart bandage to determine the uric acid (a key biomarker of wound) through biosensors. This sensor communicates with potentiostat and requests wireless information transfer via radio frequency mode or field communication with connected devices, such as smartphone, tablet computer, and other smart devices. The smart bandage governing system updates the wound healing process to the health professionals on a regular basis. Considering the advances in wound management system, the forthcoming bandages are anticipated to have better and improved healthcare applications.

8.5.6 Medical Devices

PHA polymer has been foreseen as a potential material useful in the construction of several medical devices. A number of investigations have proved its high biocompatible, biodegradable, photo-catalytic, physiochemical, and eminent mechanical characteristics (Hawa et al. 2020; Masood et al. 2015). The devices are applied in diverse medical ailments such as wound management, skin substitutes, implants, lubricant powder, tissue matrixes, bone plate, adhesion barriers, cardiovascular grafts, orthopedic pins, staples, stents, screws, sutures, surgical mesh, and microspheres (Ray and Kalia 2017). In a study a vessel stent produced by PHB/HHx has demonstrated abundant hemocompatibility and biocompatibility (Qu et al. 2006a). In addition, the prerequisite of biomaterials should be nontoxic, lack immunogenicity, and durable.

8.5.7 Industrial

The health hazards of non-biodegradable polymers have emerged in awareness throughout the world. Thereby, the scientific community focused on biodegradable polymers. Among the biopolymers, PHA may be the feasible substitute to ease the environmental burden and noninvasive applications. Advances in large-scale production of PHA have revealed the various opportunities in application arenas (Gholami et al. 2016). Many companies commercially manufacture PHAs such as BP and Metablix USA, Kaneka Japan, and Zeneca Bio Products (Vandi et al. 2018). In the near future, it is anticipated that PHA can replace harmful polymers used in various fields, particularly a short-term or one-time use application.

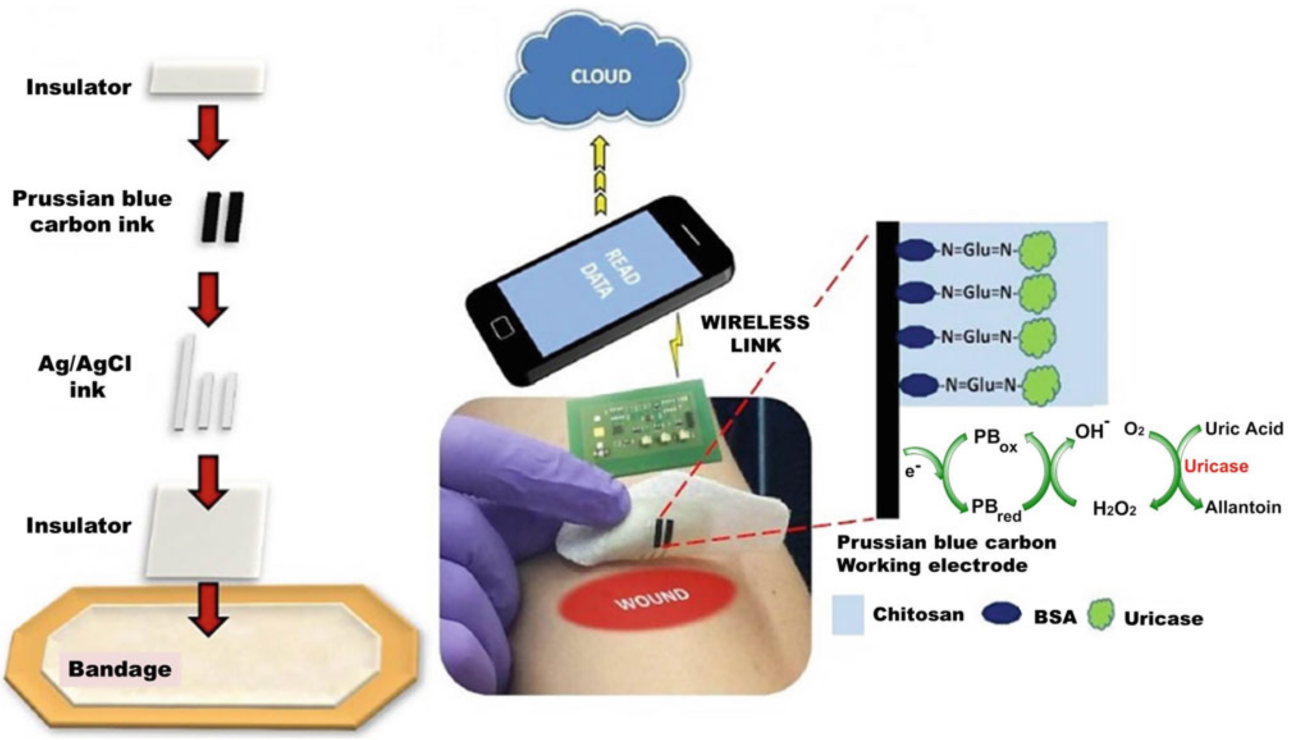


Fig. 8.7 Smart wound bandage containing wireless system to communicate with health professionals (Kassal et al. 2015)

8.6 Future Prospect

The limitation of PHA synthesis is the early deposition of PHA granules in the cells, which stops the log phase of the bacterial cell. This results in a poor quantity of cells that results in a low yield of PHA. This scenario can be achieved by increasing the quantity of cells by hindering early PHA synthesis at log phase. The medical application of PHA-based products possibly executes many functions through interdependent sensing topology in physiological conditions. Designing and engineering biosmart PHA scaffold may require expertise from diverse fields including medical doctors. In the near future, PHA-based biosmart products may be used in the treatment of countless medical ailments rather than just single-purpose or one-time use products such as drug delivery.

8.7 Conclusion

PHAs have been comprehensively investigated in several applicative researches, predominantly in modern science. It has the limitations of high production cost, toxic, and problematical refinement process including poor functionalities. To communicate these limitations, it is essential to recognize high PHA manufacturing microbes and improving the in-process methodology for procuring mass production at low budget. In addition, the metabolic modification may improve the unique monomers of PHA. A suitable biogenic extraction of PHA via macro host is required to reduce a lengthy and toxic chemical extraction of PHA.

Diversely, PHA tailoring through chemical alteration or physical blending perhaps improves the fabricated PHA mats through many advanced features. Here in this chapter, we briefed the properties of PHA and its various biomedical applications. Modification of PHA by mixing other biocompatible substances may cut the final product cost as compared to pure polymers (P(4HB)). The modified PHA-based composites have been defining significant results such as better-quality flexibility, tensile strength, biodegradation rate, thermal stability, load bearing efficacy, hydrophilicity, good rheological, functional, physiochemical, and biocompatible properties in vast biomedical applications. The various eminent properties of PHA have attracted the interest of scientists, which leads to further insight into many other medical fields.

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9

Bacterial Production of Poly-β-hydroxybutyrate (PHB): Converting Starch into Bioplastics

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Sitti Wirdhana Ahmad

Abstract

Poly-β-hydroxybutyrate (PHB) is a thermoplastic polyester accumulated intracellularly by many microorganisms under unfavorable growth conditions. The features of PHB are biodegradable and biocompatible, and the physical properties are similar to polypropylene, which has attracted industrial attention as an environmentally degradable plastic for a wide range of agricultural, marine, and medical applications and appropriate substitutes for hydrocarbon-based plastics. Starch is a renewable carbon source from plant sources available abundantly in large quantities throughout the globe and has recently been used as a carbon source for PHB production. The utilization of starch in PHB production needs enzymatic hydrolysis for starch degradation since many microorganisms do not produce these enzymes natively. This suggests there is a need for exploitation of bacterial culture for the co-production of the starch-hydrolyzing enzyme (amylolytic bacteria) as well as PHB. Some bacteria have been reported capable to convert starch into PHB directly, which are from the genus *Bacillus*. The process of PHB production from starch by amylolytic bacteria is simultaneous saccharification and fermentation (SSF). The mechanism of bacteria synthesizing PHB from starch is divided into two groups, namely, the growth-associated PHB

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synthesis and the non-growth-associated PHB synthesis. The utilization of starch for PHB production is an economic strategy to reduce production costs of PHB as well as its applications in various fields.

Keywords

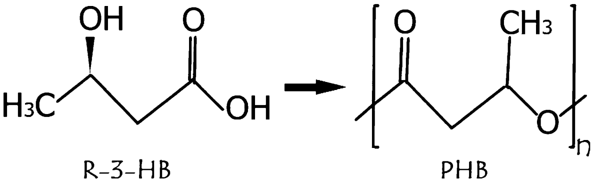
Bacterial · Bioplastic · Poly-β-hydroxybutyrate (PHB) · Starch · Biopolymer

9.1 Introduction

Polyhydroxyalkanoate (PHA) is known as bioplastics produced by microbes or also called microbial bioplastics which were investigated by Beijerinck in 1888 under a microscope as granules inside cells, whereas PHA composition was discovered by Lemoigne in 1927 (Byrom 1987). Polyhydroxyalkanoates (PHAs) are the most versatile bioplastics with properties similar to petroleum-based plastics (Anderson and Dawes 1990; Madison and Huisman 1999). As a family of biodegradable and biocompatible polyesters, PHA could be developed as environmentally friendly bulk plastics, provided the production cost is competitive (Anderson and Dawes 1990; Madison and Huisman 1999; Lenz and Marchessault 2005). Poly-β-hydroxybutyrate (PHB) is the most common PHA and was first described by Lemoigne, which is accumulated by *Bacillus megaterium* (Byrom 1987). PHB is a biopolymer synthesized by microbes and accumulated as intracellular granules and functions as carbon and energy reserves (Madison and Huisman 1999; Lenz and Marchessault 2005). This polymer was introduced as a prototype of degraded thermoplastics (Byrom 1987) that can solve the problem of plastic pollution. PHB is composed of 3-hydroxybutyrate monomers strung together by β-bonds (Fig. 9.1) (Madison and Huisman 1999; Lenz and Marchessault 2005; Zilliges and Damrow 2017). Carboxyl groups from one monomer bind to another hydroxyl monomer group with ester bonds (Anderson and Dawes 1990; Madison and Huisman 1999; Zilliges and Damrow 2017).

The advantages of PHB as a bioplastic lie in its properties such as heat-resistant, easily formed, not easily broken, and biodegradable (Anderson and Dawes 1990). PHB also has biocompatible properties of organ systems in the human body so that it is potentially used in the medical field (Madison and Huisman 1999; Lenz and Marchessault 2005). Some of the PHB bioplastic products that have been marketed under the Biopol trademark from England and Wella from Germany are combs, toothbrushes, bags, trash cans, and packaging bottles (Byrom 1987; Lee 1996). PHB

Fig. 9.1 Chemical structure of poly-β-hydroxybutyrate (PHB) and its monomer



production as bioplastics is constrained due to high production costs so that it cannot compete with synthetic plastics (Byrom 1987; Lee 1996). Therefore, various efforts are needed to reduce the cost of PHB production such as the discovery of strains of superior PHB-producing bacteria and the use of alternative carbon sources that are cheaper than glucose. Starch is an inexpensive substrate and can be generated from various agricultural wastes. Therefore, starch may be a good candidate to replace the more expensive carbon sources for the industrial production of PHB (Kim 2000; Halami 2008; Ramadas et al. 2009).

9.2 Overview of Starch as a Substrate for PHB Production

The substrates commonly used for polyhydroxyalkanoate (PHA) production are simple carbohydrates such as glucose and sucrose (Lee 1996). The substrate used for PHA production can determine the type, characteristic, quality, and quantity of polymer synthesized by microbes (Byrom 1987; Kim 2000). For example, when glucose is the sole substrate, then a polymer formed is poly-β-hydroxybutyrate (PHB), but if the substrate is a mixture of glucose and propionic acid, the product formed is a copolymer poly-β-hydroxybutyrate-co-poly-hydroxyvalerate (PHBV) (Byrom 1987). Currently, the use of inexpensive carbon sources in the biosynthesis of PHA is predicted to reduce its production cost. Some study reported that the use of complex substrates such as starch, as the carbon source for the production of PHB, can reduce its production cost since it is inexpensive and widely available to users (Kim and Chang 1998; Kim 2000; Gonzalez-Garcıa et al. 2011; Halami 2008; Yanti and Muhiddin 2016).

Starch is a polysaccharide of glucose made of two types of α-D-glucan chains, amylose and amylopectin. Starch molecules produced by each plant species have specific structures and compositions (such as the length of glucose chains or the amylose/amylopectin ratio), and the protein and fat content of the storage organs may vary significantly (Egharevba 2019). Starch is very potential as a substrate for PHB production since the monomer of starch is glucose; besides that, the carbon content is high, while the nitrogen and phosphorus levels are low (Alcazar-Alay and Meireles 2015; Yanti et al. 2009). PHB will be produced by bacteria when glucose is the sole carbon source, and the nutrients in the growth media are not balanced, namely, excessive carbon content, while other nutrient contents such as nitrogen, phosphorus, and kalium are limited (Byrom 1987; Anderson and Dawes 1990). Several types of starch can be used as substrates to produce PHB. The types of starch that have been explored as substrates to produce PHB are shown in Table 9.1. Nevertheless, before being used as a convenient carbon source for most of the known PHA-producing strains, nowadays the majority of the complex carbohydrates need treatment such as hydrolysis (Hassan et al. 1997; Kim and Chang 1998). Several PHB productions using starch as substrates perform stages of hydrolysis of starch using enzyme (Syamsu et al. 2006; Haas et al. 2008; Krueger et al. 2012) and chemical compounds (Yu 2001; Jiang et al. 2016). This pretreatment step implies an extra cost and can cause environmental problems whether chemicals

Table 9.1 Production of PHB by bacteria using various types of starch as a substrate

Starch type	Bacterial strains	PHB content (%)	References
Soluble starch	*Azotobacter chroococcum* H23	73.9	Kim (2000)
Soluble starch	*Haloferax mediterranei* ATCC 33500	60	Lillo and Rodriguez-Valera (1990)
Starch	*Saccharophagus degradans* ATCC 43961	7.12	Gonzalez-Garcıa et al. (2011)
Soluble starch	*Bacillus cereus* CFR06	48	Halami (2008)
Sago starch	*Bacillus megaterium* PSA10	85.4	Yanti and Muhiddin (2016)
Sago starch	*Bacillus cereus* PPK6	30.90	Yanti et al. (2019)
Sago starch	*Bacillus subtilis* PPK5	10.83	Yanti et al. (2019)
Canna starch	*Bacillus megaterium* PSA10	27.56	Yanti (2013)
Cassava starch	*Bacillus megaterium* PSA10	21.48	Yanti (2013)
Arrowroot starch	*Bacillus megaterium* PSA10	36.92	Yanti (2013)
Arrowroot starch	*Micrococcus* sp. PG1	16.8	Margino et al. (2014)

are used in the hydrolysis process. These problems can be overcome by finding bacterial strains that are able to hydrolyze starch and synthesize PHB simultaneously.

9.3 Poly-β-hydroxybutyrate (PHB)-Producing Microbes

Poly-β-hydroxybutyrate (PHB) is a polymer produced by microbes, mainly from the prokaryotic group (Anderson and Dawes 1990). Prokaryotic microbial groups that can accumulate PHB include Gram-negative bacteria (Byrom 1987; Sudesh et al. 2000), Gram-positive bacteria (Najimudin et al. 1997; Sudesh et al. 2000; Aslim et al. 2002; Yilmaz et al. 2005; Yanti et al. 2019), and *Cyanobacteria* (Sudesh et al. 2000). PHB-producing bacteria from the Gram-negative group are strains belonging to the genera *Alcaligenes*, *Azospirillum*, *Beijerinckia*, *Chromobacterium*, *Chromatium*, *Cupriavidus*, *Derxia*, *Ferrobacillus*, *Hyphomicrobium*, *Methylobacterium*, *Pseudomonas*, *Rhodospirillum*, *Spirillum*, *Vibrio* and *Zoogloea*. *Rhizobium*, *Rhodopseudomonas*, *Rhodospillus*, *Rhodospillillum*, *Rhodospillillum*, *Zephromogillus*, *Rhodospillum*, and *Zephromogillus* (Sudesh et al. 2000), and Gram-positive bacteria groups are strains of the genus *Actinomyces*, *Bacillus*, *Micrococcus*, *Nocardia*, and *Streptomyces* (Byrom 1987; Lenz and Marchessault 2005; Yanti et al. 2019), while strains belonging to the genus *Chlorogloea* and *Spirulina* are a member of the *Cyanobacteria* group (Byrom 1987; Lenz and Marchessault 2005).

In general, PHB-producing bacteria use simple substrates such as glucose. However, not many of these bacteria can use complex substrates such as starch to produce PHB. PHB-producing bacteria that have been used on an industrial scale such as *Cupriavidus necator* and *Alcaligenes latus* are highly dependent on glucose as a substrate in producing PHB, causing high production costs (Anderson and Dawes 1990; Lenz and Marchessault 2005). Utilization of bacteria that can use starch as a cheaper substrate to produce PHB can reduce production costs (Kim and Chang 1998; Kim 2000; Halami 2008). However, these bacteria must have amylolytic activity to use starch to produce PHB bioplastics efficiently. Therefore, searching for amylolytic bacteria producing PHB is an effort that can be done to reduce the production cost of PHB.

Several studies have been conducted to explore indigenous amylolytic bacteria that can be utilized in producing PHB using starch as a substrate (Halami 2008; Margino et al. 2014; Yanti et al. 2019). Margino et al. (2000) succeeded in isolating amylolytic bacteria that accumulated PHB from tapioca industrial waste and were identified as genera *Bacillus* and *Pseudomonas*. Halami (2008) obtained indigenous bacterial isolates that were able to produce PHB from starch substrates and were identified as *Bacillus cereus*. Yanti et al. (2019) also obtained indigenous bacterial isolates from the sago starch processing area that were able to produce PHB from sago starch substrates and were identified as members of the genus *Bacillus*. In general, bacteria that can convert various types of starch into bioplastic PHB directly are the genus *Bacillus*, namely, from the species *Bacillus megaterium*, *Bacillus subtilis*, and *Bacillus cereus* (Halami 2008; Yanti et al. 2019). Besides the genus *Bacillus*, a bacterial strain of the genera *Azotobacter* (Kim 2000), *Haloferax* (Lillo and Rodriguez-Valera 1990), *Saccharophagus* (Gonzalez-Garcıa et al. 2011), and *Micrococcus* (Margino et al. 2014) was reportedly capable of producing PHB from starch (Table 9.1). Out of these, *Bacillus* spp. are found to be more efficient for PHB production due to their higher stability and reproducibility under environmental stress (Shivalkar and Prabha 2017). These bacteria are very potential to be used to produce PHB bioplastics using various types of starch industrially.

9.4 PHB Detection

Poly-β-hydroxybutyrate (PHB) is a polymeric ester which functions as an energy and carbon reserve in prokaryotic cells. PHB exists as discrete inclusions or granules in the cell (Ostle and Holt 1982). To start an extensive search of bacterial population of PHB producers, it was necessary to develop simple qualitative and quantitative methods of PHB content estimation in living bacterial cells. PHB detection in bacterial cells is done by the staining method. Sudan Black, Nile blue, and Nile red are stains that can be used to detect PHB granules (Ostle and Holt 1982; Amara 2008). The three stains have a high affinity for PHB granules (Ostle and Holt 1982), so they are good for screening for PHB-producing bacteria. Redzwan et al. (1997) reported the use of Sudan Black B staining technique as a first-line screening for PHB-producing bacteria from nature, needed to screen various bacterial collections in a short time by using special staining for the detection of PHB granules. Hartman (1940) was the first to suggest the use of Sudan Black B, as a bacterial fat stain. The lipophilic stain Sudan Black B has long been regarded as a dye with particularly high affinity for PHAs (Murray et al. 1994). The Sudan Black B staining was used as the first line of qualitative observation of PHB production for the bacterial species as also suggested by certain workers (Phanse et al. 2011). Different bacterial species such as *Bacillus subtilis* NRRL-B-941, *Bacillus licheniformis* B-NRRL 1001, *Bacillus cereus* NRRL-B-3711, *Bacillus megaterium* NRRL-B-3712, *Bacillus thuringiensis* 798 (Phanse et al. 2011; Asad et al. 2016), and *Bacillus megaterium* PSA10 (Yanti et al. 2009) were screened based on qualitative tests using a Sudan Black B staining. In several studies, the detection of PHB accumulation using Sudan Black staining has been carried out macroscopically, namely, the coloring of bacterial colonies on the agar plate (Asad et al. 2016; Yanti et al. 2019). Sudan Black staining method was microscopically performed by coloring the bacterial cells using Sudan Black solution 0.2% (w/v) in 70% ethanol for 10 min and then washing with xylene for 10 s and then painting with safranin. Observation using a phase-contrast microscope has shown that PHB granules in cells are blackish blue while vegetative cells are red (Fig. 9.2a). PHB detection using Sudan Black staining macroscopically is done by staining bacterial colonies grown on an agar plate medium containing starch 1% (w/v) as carbon source using Sudan Black solution 0.02% (w/v) dissolved in 96% ethanol for 10 min and then washing with 100% ethanol. The dark-blue-colored colonies were taken as positive for PHA production (Fig. 9.2b) (Yanti et al. 2019).

Besides Sudan Black stain, Nile blue A is another stain for the detection of PHB granules in bacteria and is, in fact, superior to Sudan Black B since it is not as easily washed from the cell by decolorization procedures (Ostle and Holt 1982; Mascarenhas and Aruna 2017). Ostle and Holt (1982) advocated the use of Nile blue A, a water-soluble basic oxazine dye that has a greater affinity and higher specificity than Sudan Black for PHB detection, and gives a bright orange fluorescence on exposure to ultraviolet light. The oxazine form of the dye Nile blue is responsible for the fluorescent staining of PHB (Ostle and Holt 1982; Mascarenhas and Aruna 2017). Other inclusion bodies, such as glycogen and polyphosphate, do not stain with Nile blue A, thus emphasizing its usefulness. PHB detection using a Nile blue staining could be done microscopically and macroscopically. The method of observing PHB granules in bacterial cells (microscopically) uses Nile blue stain as follows: spread bacterial cell preparations on an object glass and then drip with Nile blue solution and then heat at a temperature of 55 °C using a hot plate for 10 min. After staining, the slide preparation is washed with running water to remove excess dyes and then washed with 8% (v/v) acetic acid for 1 min. The preparations are washed with running water and then dried. Dry preparations were observed using a fluorescence microscope, and PHB granules in bacterial cells will fluoresce when observed with a fluorescence microscope (Ostle and Holt 1982) (Fig. 9.2c). Kitamura and Doi (1994) first demonstrated the viable colony method on agar plates; they induced the isolates to accumulate PHA by culturing in E2 medium containing 2% (w/v) glucose before Nile blue A staining. The PHA-accumulating colonies, after Nile blue A staining, showed bright orange fluorescence on irradiation with

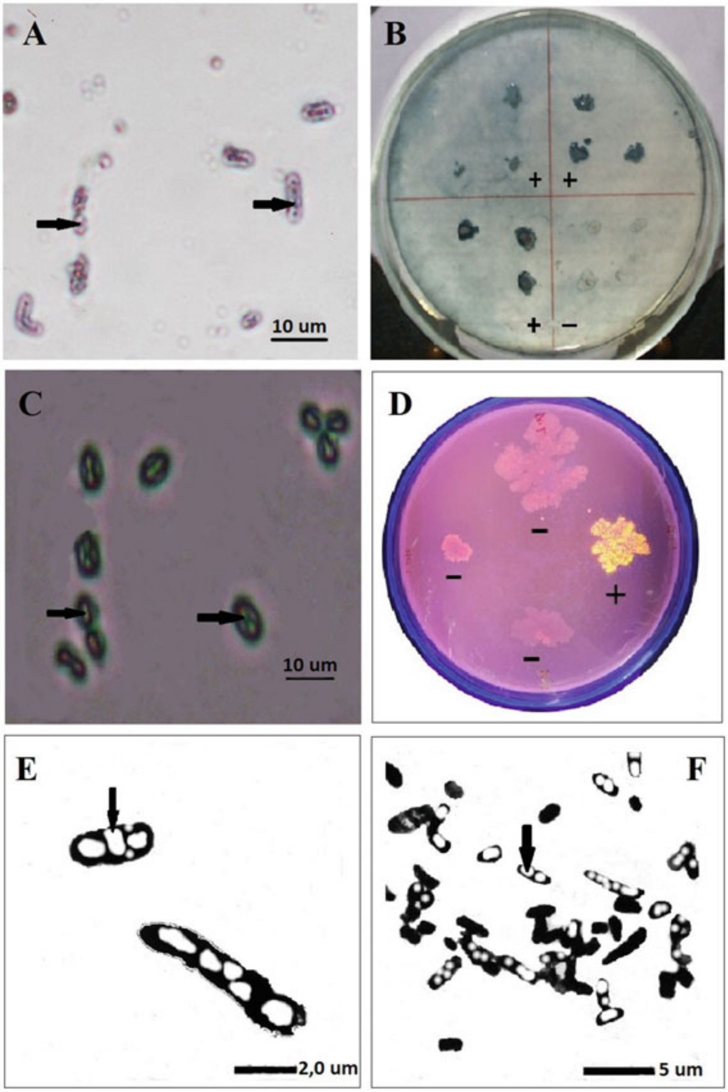


Fig. 9.2 Detection of PHB granule accumulation in bacteria. (**a**) Micrographs showing PHB granule with Sudan Black staining under a phase-contrast microscope. (**b**) PHB-accumulating colonies with Sudan Black staining (+, positive PHB; −, negative PHB). (**c**) Micrographs of PHB granule with Nile blue under a fluorescence microscope. (**d**) The PHB-accumulating colonies, after Nile blue A staining, showed bright orange fluorescence on irradiation with UV light (+, positive PHB; −, negative PHB). (**e**, **f**) PHB granules under the transmission electron microscope. Black arrows indicate PHB granules inside the cells

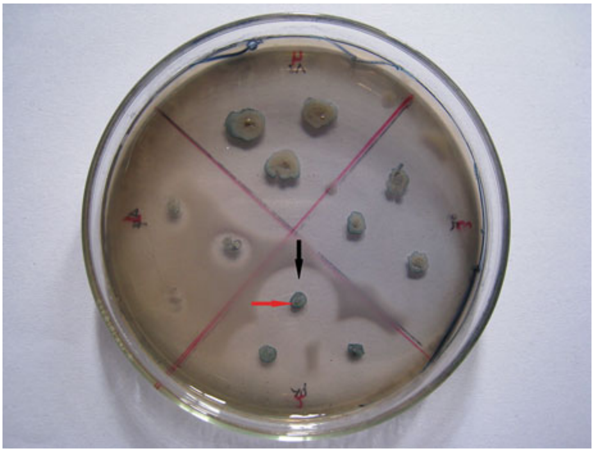


Fig. 9.3 Detection of amylolytic bacterial colonies and PHB-producing on starch agar media. The black arrows indicate clear zones formed by starch hydrolysis activity (amylolytic activity), and the red arrows indicate the blackish-blue colonies that accumulate PHB

UV-light at 312 wavelengths (Fig. 9.2d), and their fluorescence intensity increased with an increase in PHA content of the bacterial cells (Kitamura and Doi 1994). PHB granules formed intracellularly were observed using transmission electron microscopy (TEM). Observation of PHB granules using TEM will clarify PHB in cells. Sample preparation for the observation of PHB granules with TEM was carried out based on the method described by Rodríguez-Contreras et al. (2013). The sample was fixed with a mixture of 2% (v/v) glutaraldehyde solution, 3% (w/v) paraformaldehyde, 5% (w/v) sucrose, and 0.1 mol/L sodium cacodylate. Furthermore, the bacterial cell was dehydrated using an ethanol solution and then planted in a polymerized polymer at 60 °C overnight. Bacterial cells embedded in the resin are then sliced using ultra-microtomes with a thickness of about 70 nm. After that, the samples were examined using a transmission electron microscope. PHB granules in bacterial cells observed with a TEM microscope are shown in Fig. 9.2e, f. Exploration of amylolytic bacteria (the ability to hydrolyze starch), as well as PHB-producing, is needed to find bacteria that can convert starch into bioplastic PHB directly. Amylolytic bacterial screening and PHB-producing can be demonstrated as the viable colony method on agar plates containing starch 1% (w/v) as carbon sources. Detection of amylolytic ability, using Lugol's iodine whereas PHB accumulation using Sudan black staining (Yanti et al. 2019). The clear zone formed around the bacterial colonies on starch agar media indicates amylolytic activity (starch hydrolysis), while the blackish-blue color of the colonies indicates the bacterial cells can accumulate PHB (Fig. 9.3).

9.5 Downstream Processing of PHB (Recovery and Purification)

PHB is synthesized in bacterial cells, so PHB must be extracted from cells to be purified before being used as bioplastics. Several solvent extraction processes have been developed to recover PHB from biomass. For example, PHB can be extracted from bacterial cells including various acids (HCl, H_2SO_4), alkalies (NaOH, KOH, and NH_4OH), and surfactants namely dioctyl sulfosuccinate sodium salt (AOT), hexadecyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), polyoxyethylene-p-tert-octylphenol (Triton X-100), and polyoxyethylene (20) sorbitan monolaurate (Tween 20) (Choi and Lee 1999) and chloroform (Jacquel et al. 2008). SDS is an efficient chemical for PHB isolation from recombinant *E. coli*, but the price is expensive and it has a waste disposal problem (Choi and Lee 1999). NaOH and KOH were also efficient and economical for the recovery of P(3HB) (Choi and Lee 1999). A less complex procedure is the use of a differential digestion method employing sodium hypochlorite. Although simple and effective, this method has been avoided because sodium hypochlorite can cause a decrease in the molecular weight of PHB as much as 50% (Lee 1996). Ramsay et al. (1990) reported that by optimizing the conditions of sodium hypochlorite digestion and by balancing the ratio of hypochlorite to non-PHB biomass, PHB of 95% purity with an average molecular weight of 600,000 was recovered from *Alcaligenes eutrophus*. PHB is hydrophobic, while lyophilized cells are hydrophilic. When PHB is isolated from the cell by the action of hypochlorite, it will immediately migrate to the chloroform phase avoiding severe degradation. Chloroform can, at least partially, protect the PHB molecules from further destructive action of the hypochlorite (Ramsay et al. 1990). Sodium hydroxide (NaOH) can be used as a substitute for sodium hypochlorite in the PHB extraction process because it can obtain up to 97% PHB. Another advantage of sodium hydroxide compared to sodium hypochlorite is it is inexpensive and environmentally friendly (Choi and Lee 1999).

9.6 Metabolism of Poly-β-hydroxybutyrate (PHB)

9.6.1 Synthesis of PHB

The mechanism of PHB synthesis from starch by bacteria occurs in two stages, namely, the hydrolysis of starch to glucose and the synthesis of glucose into PHB. The one-step bioconversion of starch into PHB can be done by using microorganisms that are able to degrade or digest starch into glucose and then the fermentation glucose into PHB (Yanti et al. 2013; Yanti and Muhiddin 2016). Bacteria that can convert starch into bioplastic PHB directly in one stage are amylolytic bacteria, which can hydrolyze starch into simple sugars and at the same time are able to synthesize PHB also (Halami 2008; Yanti et al. 2013; Yanti and Muhiddin 2016). This process is called as simultaneous saccharification and fermentation (SSF). In the process of SSF, the glucose produced by the hydrolyzing

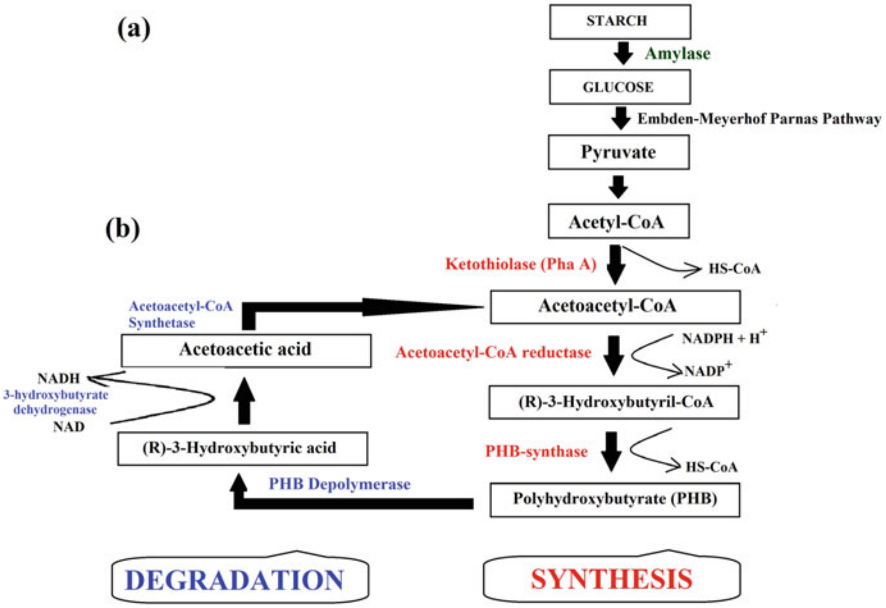


Fig. 9.4 Metabolism of PHB from starch. (**a**) Mechanism of starch hydrolysis to glucose. (**b**) Mechanism of PHB synthesis and degradation

enzymes is consumed immediately by the fermenting microorganism present in the culture (Taherzadeh and Karimi 2007). SSF process has many advantages to use as a fermentation strategy; they are the risk of contamination is low and the number of vessels required is reduced, resulting in lower capital cost of the process (Taherzadeh and Karimi 2007).

The pathway of PHB synthesis from starch by amylolytic bacteria gets completed in several steps. Starch is hydrolyzed into glucose by the amylase enzyme, and then the glucose is converted into pyruvate via the Embden-Meyerhof-Parnas pathway for subsequent entry into the synthesis of PHB. PHB synthesis is carried out by a series of enzymes, namely, (1) *β-ketothiolase (acetyl-CoA acyltransferase)* which catalyzes the dimerization of acetic acid CoA (acetyl-CoA) derivatives into acetoacetyl-CoA, (2) *acetoacetyl-CoA reductase* that catalyzes the hydrogenation of acetoacetyl CoA that becomes [R]-3-hydroxybutyryl-CoA which is a PHB monomer, and (3) PHB synthase that catalyzes the polymerization of PHB monomers to PHB (Braunegg et al. 1998; Lenz and Marchessault 2005). The mechanism of PHB synthesis from starch is presented in Fig. 9.4. Under conditions of unbalanced growth (excessive carbon but other nutrients are limited), NADH oxidase activity in the Krebs cycle decreases, thereby increasing the amount of NADH (Byrom 1987; Braunegg et al. 1998). The increase in NADH will inhibit the activity of citrate synthase so that acetyl-CoA and oxaloacetate (OAA) are not converted into citrate and free CoA and cause acetyl-CoA acetyltransferase (β-ketothiolase) to become active to condense acetyl-CoA to acetoacetyl-CoA which is the initial compound of the polymerization of PHB (Doi et al. 1992; Braunegg et al. 1998). As long as the condition of the medium has not changed, microbes remain to accumulate PHB in their cells, but if the carbon concentration in the medium declines, the microbes will degrade PHB polymers (depolymerization) to obtain the energy needed for growth (Byrom 1987). As a result, the amount of PHB in the cell decreased (Byrom 1987; Brandl et al. 1990).

9.6.2 Degradation of PHB

The enzymes that play a role in the degradation of PHB polymers are (1) *depolymerase* or *hydrolase*, which is an enzyme that hydrolyzes PHB granules to [R]-3-hydroxybutyric acid; (2) *specific dehydrogenase*, which is an enzyme that converts [R]-3-hydroxybutyric acid to acetoacetic acid; and (3) *acetoacetyl-CoA synthetase*, which is an enzyme that converts acetoacetic acid into acetoacetyl-CoA (Lenz and Marchessault 2005). The mechanisms of PHB degradation are presented in Fig. 9.4. The PHB synthesis and degradation process are influenced by nutrient content and incubation time. Margino et al. (2000) and Yanti et al. (2013) reported that *Alcaligenes eutrophus* and several types of *Bacillus* accumulate PHB in a medium that is limited in nitrogen in an incubation period of 48–72 h. Therefore, to obtain PHB with the best quality and maximum amount, it is necessary to have the right and specific nutrient content and incubation time for each type of microbes.

9.7 Fermentation Process

Bacteria that are used for the production of PHB can be classified into two groups, depending on the culture conditions to PHB accumulation. The first group of bacteria accumulating the polymer PHB during the stationary growth phase (non-growth-associated) requires limitation of essential nutrients such as nitrogen and oxygen and the presence of excess carbon source for the efficient synthesis of PHB. The representative bacteria belonging to this group include *Alcaligenes eutrophus* (Lee 1996), *Bacillus subtilis* 25, and *Bacillus megaterium* 12 (Yuksekdag et al. 2004) and *Bacillus subtilis* PPK5 (Yanti et al. 2013). On the other hand, the second group of bacteria accumulating the polymer during the growth (exponential) phase (growth-associated) does not require nutrient limitation for PHB synthesis. Some of the bacteria included in this group are *Alcaligenes latus* (Lee 1996), *Bacillus mycoides* (Thakur et al. 2001), *Bacillus sphaericus* NCIM 5149 (Ramadas et al. 2009), and *Bacillus megaterium* PSA10 (Yanti et al. 2013, 2020). The culture conditions required for PHB biosynthesis are important criteria to be taken into consideration for the development of cultivation techniques used in the large-scale production of PHB (Chee et al. 2010).

The fermentation process of PHB using starch by amylolytic bacteria consists of two mechanisms. In the first group, amylolytic bacteria hydrolyze the starch to glucose which is then used for growth that synthesizes PHB directly so that glucose

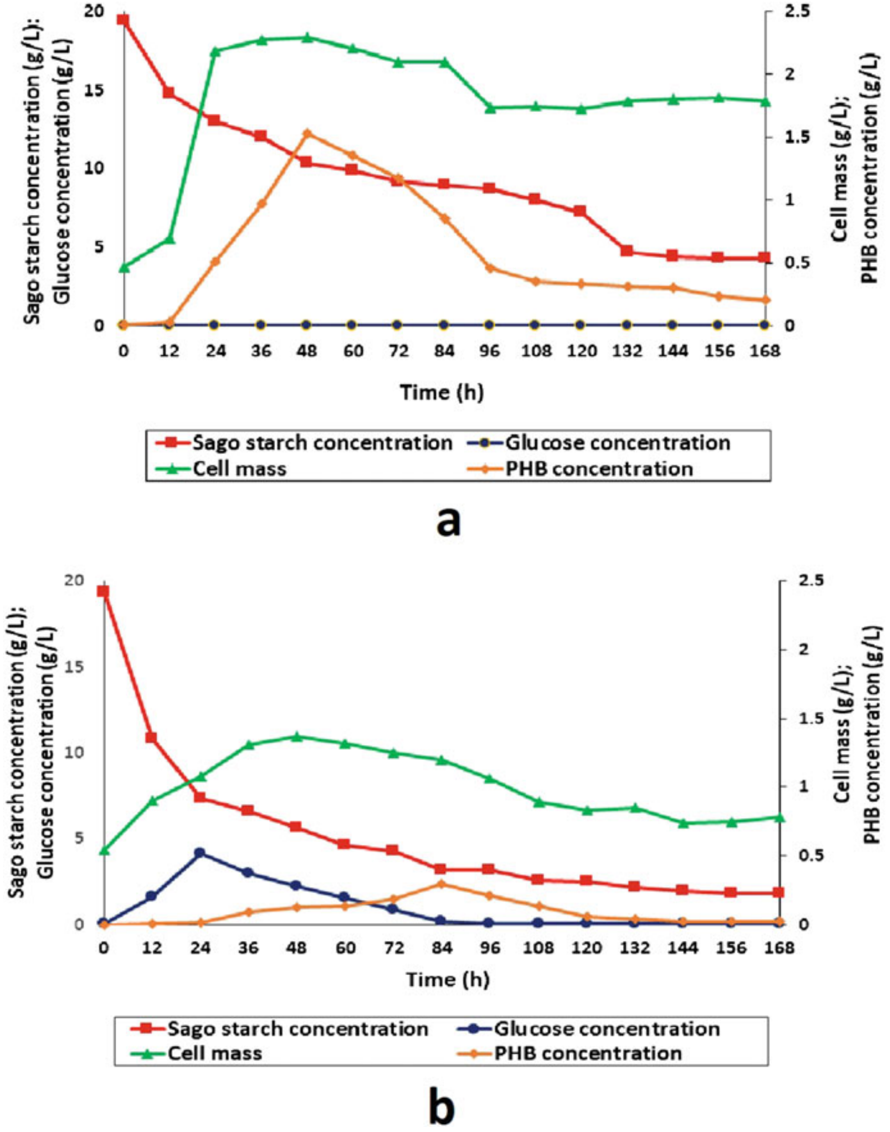


Fig. 9.5 PHB fermentation process from sago starch. (**a**) Bacteria growth-associated, (**b**) bacteria non-growth-associated

does not accumulate in the fermentation media. In this mechanism, bacteria synthesize PHB together with their growth (growth-associated) (Fig. 9.5a). Bacteria that belong to this group are *Bacillus megaterium* PSA10 (Yanti et al. 2013, 2020). In the second group, amylolytic bacteria hydrolyze starch to glucose, and then glucose is accumulated in the fermentation media. In this process, PHB began synthesized at the late exponential phase or in early stationary phase of growth after the high glucose levels in the medium, and PHB synthesis is increased after growth decline (non-growth-associated) (Fig. 9.5b). Bacteria included in this group are *Bacillus subtilis* PPK5 (Yanti et al. 2013, 2019).

9.8 Characteristics of PHB

PHB is an aliphatic homopolymer that has thermoplastic properties with good mechanical properties and has a similar characteristic to polypropylene (PP). A comparison of physical characteristics between PHB and polypropylene is shown in Table 9.2. PHB is an intracellular product, if PHB still inside the cell are amorphous, but after going through an extraction process using an organic solvent, PHB will change into a highly crystalline. The high crystallinity causes PHB to be a rigid but brittle material. The PHB fragility caused is not resistant to pressure. PHB melting temperature (175 °C) which is approaching its thermal degradation temperature (200 °C) caused a limitation in the processing process (Madison and Huisman 1999). According to Kim and Rhee (2003), this weakness can be improved by copolymerizing poly-β-hydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) into a copolymer of poly-hydroxybutyrate-co-polyhydroxyvalerate (PHB-co-HV) that is more flexible and has low process temperature. PHB characteristics such as crystallinity and tensile strength, depending on the molecular weight of the polymer. The amount of molecular weight is influenced by the strain of microorganism used, the cultivation conditions, and the purity of PHB (Pena et al. 2014). The average molecular weight of PHB varies from 2 to 4 $\times 10^3$ kDa. The molecular weight depends on the ability of microbes to accumulate the produced polymer, conditions of growth, and extraction method (Rajan et al. 2018). Bourque et al. (1995) state that the molecular weight of PHB can be reduced during the polymer processing process. Lafferty et al. (1988) added that the reduction in PHB molecular weight can occur throughout the extraction process from biomass.

According to Hrabak (1992), PHB has polypropylene-like characteristics with three unique properties, namely, thermoplastic, 100% waterproof, and 100% biodegradable. PHB has several superior characteristics such as water vapor resistance and insoluble in water, and these characteristics distinguished the PHB with other biodegradable plastics (Lenz and Marchessault 2005). PHB also has good impermeability to oxygen. PHB can dissolve in various solvents such as chloroform,

Table 9.2 Comparison of physical characteristics between PHB and polypropylene

Characteristics	PHB	Polypropylene (PP)
Melting point, Tm (°C)	175	176
Glass transition temp., Tg (°C)	5	−10
Crystallinity (%)	80	70
Tensile strength (MPa)	40	34
Elongation at break (%)	6	400

Table 9.3 The solubility of PHB in various solvents

High solubility	Moderate solubility	Not dissolved
Chloroform	Dioxane	H_2O
Dichloromethane	Octanol	Methanol
Di-, tri-, and tetrachloroethane	Toluene	Ethanol
Ethylene carbonate	Pyridine	1-Propanol
Propylene carbonate		2-Propanol
Acetic acid		Benzene
Alcohol (>3 C atoms)		Ethyl acetate
		Ethylmethylketone
		Butyl acetate
		Tributyl citrate
		Hexane

methylene chloride, ethylene chloride, pyridine, or dichloromethane/ethanol mixture (Choi and Lee 1999). The solubility of PHB in some solvents is shown in Table 9.3.

9.9 Applications of Bioplastic PHB

PHB can be applied as plastic because it is biodegradable, thermoplastic, and piezoelectric and has the ability to depolymerize PHB into a D-3-hydroxybutyric acid monomer (Lafferty et al. 1988). Generally, PHB application is divided into three areas, namely, medical and pharmaceutical, agricultural, and packaging (Mathuriya and Yakhmi 2017; Koller 2018). Some examples of practical applications of PHB are shown in Table 9.4. PHB polymers produced by Gram-

Table 9.4 Practical applications of PHB

Medical and pharmaceutical
1. Surgical operation requirements: sewing thread, pins, swabs
2. Wound dressing
3. Installation of blood vessels and body tissues (due to the ability of PHB depolymerization to become a D-3-hydroxybutyric acid monomer)
4. Installation of bones and bone plates
5. Stimulation of bone growth (because PHA has piezoelectric properties)
6. Drug delivery system
Agricultural
1. Biodegradable carrier of active ingredients on herbicides, fungicides, insecticides, or fertilizers (due to the ability of degradation in the soil)
2. Seedling container
3. Biodegradable matrix for drugs in the veterinary field
Packaging
1. Container, bottle, bags, and film packaging
2. Disposable materials such as baby diapers and sanitary napkins

positive bacteria have the potential to be used for medical equipment. According to Valappil et al. (2007), the use of Gram-positive bacteria as a producer of PHB for medical purposes has advantages compared to Gram-negative bacteria. Gram-negative bacteria have lipopolysaccharides (LPS) in their cell walls which can cause immunogenic reactions, while Gram-positive bacteria do not have LPS (Valappil et al. 2007).

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Halophilic Microorganisms as Potential Producers of Polyhydroxyalkanoates

10

Angelina Stanley, Sarma Mutturi, and S. V. N. Vijayendra

Abstract

Polyhydroxyalkanoates (PHA), a kind of storage energy produced by several microorganisms, have gained much attention over the years as a suitable alternate to non-biodegradable plastics. These are biodegradable, biocompatible, and non-toxic polymers, which are being used in various industrial and biomedical applications. PHA can also help to mitigate environmental pollution by replacing the non-biodegradable petroleum-derived plastics. However, the drawback for the use of PHA is its high cost of production at industrial scale. Halophilic microbes inhabiting diverse saline environment have unique physiology and adaptability that make them useful tools for exploring metabolites for industrial applications. PHA can be produced by halophilic microorganisms, which are less explored for their potential. PHA production by halophiles has certain advantages. As halophiles grow at higher salt concentrations, the growth of non-halophilic microorganisms can be prevented, thus eliminating the need for strict sterile operating conditions, and this reduces the cost of production of PHA. In addition, the polymer could be recovered by the hypo-osmotic shock of the cells enabling easy downstream processing. Reports are available on the various advances being made in the production of PHA in an economically feasible manner. These include optimization strategies focusing on halophilic microorganisms, such as supplying renewable carbon substrates, developing better bacterial strains, optimization of fermentation processes, engineering new pathways, etc. Thus, exploring new possibilities for improved PHA production in halophilic microorganisms, which are potential candidates for bioproduction in

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industrial setups, is the need of the hour. This chapter focuses on production of PHA and its optimization along with various applications of PHA.

Keywords

Polyhydroxyalkanoates · *Halomonas* · Biodegradable plastics · Fed-batch fermentation · Applications · Upstream processes

10.1 Introduction

Polyhydroxyalkanoates (PHA) are biodegradable polymers produced by many bacterial genera and studied by researchers worldwide. Owing to the desirable properties of PHA being biodegradable and biocompatible, they serve as an alternative to conventional plastics (Meng et al. 2014). The PHA molecules are thermoplastic and elastomeric in nature. It is similar to polypropylene (PP) and low-density polyethylene (LDPE) (Kunasundari 2011). An added advantage of PHA polymers is their complete degradability by many microorganisms into carbon dioxide and water (Jendrossek 2009). Hence, they are environment-friendly and can be used in various industrial applications (Keshavarz and Roy 2010). PHA are polyesters produced by several microorganisms during unfavorable growth conditions as carbon and energy reserves. These are accumulated as intracellular granules when excess carbon source and growth-limiting essential nutrient (N, P, and K) are present in their environment (Rehm 2006). Microorganisms can accumulate PHA up to 80–90% of their biomass grown under various carbon sources (Kim et al. 1994; Yu and Stahl 2008). There are over 150 copolymers of PHA synthesized by bacteria based on the carbon source or precursors utilized (Braunegg et al. 2004). However, poly(3-hydroxybutyrate) (PHB) is the most common polymer produced by the majority of bacteria and is widely studied. PHB produced by *Bacillus megaterium* was first discovered by Lemoigne in 1926 (Lemoigne 1926). Among the microorganisms capable of accumulating high amounts of PHA (up to 80%) are *Alcaligenes latus*, *Ralstonia eutropha*, *Pseudomonas putida*, and recombinant *Escherichia coli* (Chen 2009).

The mechanical properties of PHA vary from rigid plastics to elastic rubbers. These properties are affected by the amount and compositions of monomers (Loo and Sudesh 2007). PHA polymers are biodegradable, thermoplastic, elastomeric, piezoelectric, perfectly isotactic, or optically active, having only the R-configuration. These are insoluble in water but soluble in chloroform and other chlorinated solvents. These are hydrophobic and relatively resistant to hydrolytic degradation and stable and inert in the air. PHA molecules are biocompatible as they are non-toxic, having high purity within the cell. The PHA produced by *Halomonas* sp. was characterized recently (Stanley et al. 2020). PHA molecules are resistant to UV degradation but have poor resistance to acids and bases. PHA sinks in water and thus facilitates anaerobic biodegradation in sediments. They are also less “sticky” when melted compared to traditional polymers (Hazer and Steinbuchel 2007; Laycock et al. 2013; Bugnicourt et al. 2014). Thus, petroleum-based plastics have been replaced with PHA polymers and have found various applications in the field of industry, medical aids, and agriculture. PHA in the form of nanocomposites and chiral monomers are used in pharmaceuticals. Other novel applications are being explored in recent years. However, the major drawback of the application of PHA at the industrial level is its high cost of production. Nevertheless, attempts are being made to make the PHA production economically feasible at the industrial scale. The use of low-cost carbon substrates from agro-industrial wastes, using recombinant or mixed microbial cultures, using extremophiles, and different fermentation strategies are possible ways to tackle this issue (Ienczak et al. 2013).

Because of the several potentials, extremophilic microorganisms draw our attention to explore their unique metabolic machinery for biotechnological processes. Microbes with unique physiology and adaptability found in diverse ecological niches are useful tools for exploring metabolites for industrial applications. Among such microorganisms of biotechnological importance are halophilic microorganisms. They are extremophiles that grow in a wide range of saline environments and require salt for growth. The optimal sodium chloride (NaCl) concentration ranges from 0% to 25%, and based on the salt requirement, halophiles are classified as slight, moderate, and extreme halophiles (Ventosa et al. 1998). Reports are available on metabolites produced by halophiles that are of industrial importance such as enzymes, compatible solutes, PHA, exopolysaccharides, β-carotene, and biofuels (Oren 2010). Research on PHA-producing halophilic microorganisms is less explored. The advantage of halophiles, as PHA producers, is that the growth of non-halophilic microorganisms can be prevented. Contamination is controlled since halophiles grow at higher salt concentrations. As a result, there is no need for stringent sterile operating conditions and a reduction in cost in the overall process. Another advantage is that the polymer could be recovered by the hypo-osmotic shock of the cells enabling easy downstream processing (Quillaguamán et al. 2010).

10.2 Halophilic Microorganisms

Halophilic microorganisms are found in extreme environments such as saline and hypersaline habitats. Along with high salinity, they are also exposed to low oxygen concentrations, high or low temperatures, alkaline conditions, low nutrient availability, solar radiations, and the presence of heavy metals and other toxic compounds, which are based on the geographical area (Rodriguez-Valera 1988). Salt is a natural requirement for all life forms. However, halophiles have a higher requirement that distinguishes them from others. Halophiles require salt (NaCl) for their growth and exhibit a physiological response to varying salt concentrations. Halophiles are classified as slight halophiles (marine microorganisms) that require 0.2–0.5 M NaCl, moderate halophiles that require 0.5–2.5 M NaCl, and extreme halophiles that require 2.5–5.2 M NaCl for their growth, whereas the non-halophiles can grow in salt concentrations less than 0.2 M NaCl, and the halotolerant bacteria can grow in the absence of NaCl and can tolerate a wide range of salt concentrations (Kushner and Kamekura 1988).

10.2.1 Habitat and Physiological Adaptation of Halophiles

Halophiles occupy a wide range of saline environments, such as natural brines in the coastal and deep-sea pools, salt mines, solar salterns, and soils. They also inhabit salted products like salted fish or meats and fermented food (DasSarma et al. 2010). Microorganisms living in high salt concentrations are deleterious, which causes water loss from the cells. Thus, halophilic organisms have adapted two mechanisms to prevent osmotic stress. One mechanism is the accumulation of compatible solutes to maintain low salt concentration. Compatible solutes can be either synthesized or taken up from the medium directly. Such a mechanism enables them to grow in a wide range of salt concentrations (Oren 2008; Quillaguamán et al. 2010). The other mechanism is 'salt-in' method to maintain by accumulating equal amounts of KCl to balance the extracellular NaCl concentrations. Their intracellular enzymes are also adapted to function in the presence of such high intracellular salt concentrations by having acidic proteins (Ventosa et al. 1998).

10.2.2 Diversity of Halophiles Accumulating PHA

Halophilic microorganisms are distributed among the three domains of life, viz., *Archaea*, *Bacteria*, and *Eukarya*. The halophilic bacteria and eukaryotes that can grow in low salinity are considered to be slight to moderate halophiles, whereas archaea include the extreme halophilic microorganisms that exist at higher salinity (DasSarma et al. 2010). Most studies reported on the PHA production are focused on non-halophilic counterparts than on halophilic microorganisms (Salgaonkar and Bragança 2017). A few reports on the PHA-accumulating halophilic microorganisms are from the members of the families *Halobacteriaceae* and *Halomonadaceae* that belong to the domains *Archaea* and *Bacteria*, respectively (Quillaguamán et al. 2010). It include some well-studied halophiles such as the moderate halophilic bacteria *Halomonas* spp. (Quillaguamán et al. 2010; Salgaonkar et al. 2013a) and in *Archaea*, such as *Haloferax mediterranei* and *Haloarcula marismortui* (Litchfield 2011). An extensive review is available on various halophilic bacteria and archaea with PHA-producing capabilities utilizing cheap carbon substrates from agro-industrial wastes that emphasize the importance of industrial working conditions for the cost-effective process (Koller 2017; Salgaonkar and Bragança 2019). They have an advantage of a minimum risk of contamination, and PHA production can be carried out in unsterile conditions (Don et al. 2006; Tan et al. 2011; Yin et al. 2015). Moreover, the downstream processing of the PHA granules can be done by osmotic lysis of cells, which is cost-effective (Selvakumar et al. 2011).

10.3 PHA Production by Halophilic Microorganisms

Halophilic microorganisms are considered as cell factories for the production of PHA because of the several advantages they possess (Mitra et al. 2020). The studies related to PHA production in halophiles are basically among the members of the families *Halobacteriaceae* and *Halomonadaceae* belonging to the domains *Archaea* and *Bacteria*, respectively (Quillaguamán et al. 2010). An extensive review by Koller (2017) is available on PHA production by halophilic archaea utilizing cheap carbon substrates that can be used at industrial working conditions for the cost-effective process, thus exploring the versatile metabolic machinery of these halophiles for PHA production. A list of halophilic bacteria and archaea that accumulate PHB and copolymers using different carbon sources is provided in Table 10.1.

10.3.1 PHA Production by Halophilic Bacteria

The halophilic and halotolerant bacterial species are found in the family *Halomonadaceae*, which comprises nine genera and more than 65 different species. PHA accumulation in halophilic bacteria has been recognized as a useful phenotypic marker that helps differentiate species from each other (Mata et al. 2002). A majority of the PHA accumulation has been reported in species of the genus *Halomonas*. *Halomonas boliviensis* is most extensively studied and has shown to accumulate PHB with a yield of 50–56 wt% using hydrolyzed starch (Quillaguamán et al. 2010; Saharan et al. 2014). Some of the *Halomonas* species can co-produce PHB and ectoines, such as *H. elongata* co-produced ectoine with 55% of PHA utilizing glucose in the fed-batch cultivation (Mothes et al. 2008). In addition, the moderate halophilic bacteria, *Halomonas boliviensis*, co-produced ectoine and PHB of 68.5% of cell dry weight in a fed-batch culture utilizing glucose (Guzmán et al. 2009). Therefore, the co-production of PHA and compatible solutes in halophilic bacteria is a useful tool to reduce the production cost of these economically important products.

The haloalkaliphilic bacterium, *Halomonas* sp. TD01, accumulated PHB up to 80% of the biomass on glucose salt medium and showed great potential for low-cost PHA production (Tan et al. 2011). In our study, a native marine isolate, *Halomonas venusta* KT832796 strain (Fig. 10.1), was capable of accumulating PHB from glucose up to 88% of biomass by the fed-batch process using different feeding strategies (Stanley et al. 2018). Therefore, nutrition optimization and process parameters play a key role in enhancing the PHA productivity.

The PHA accumulation in *Halomonas* sp. using different carbon sources has been reported indicating the polymer synthesis ability using different carbon sources. *Halomonas campisalis* MCM B-1027 accumulated PHA to the tune of 56.23% of dry cell weight (DCW) with maltose as a carbon source (Kshirsagar et al. 2012). *Halomonas profundus* produced PHB and PHB-co-HV from various carbon substrates with fatty acids (Simon-Colin et al. 2008). Hydrolyzed wheat bran was used as a cheap carbon source for PHA production by *H. boliviensis* (Van-Thuoc

Table 10.1 PHA accumulation in halophilic bacteria and archaea

Organism name	Substrate	PHA type	PHA (%)	References
Halophilic bacteria				
Halomonas boliviensis	Hydrolyzed starch	PHB	50–56	Quillaguamán et al. (2010), Saharan et al. (2014)
H. boliviensis	Glucose	PHB	68.5	Guzmán et al. (2009)
H. elongata	Glucose	PHB	55	Mothes et al. (2008)
Halomonas sp. TD01	Glucose	PHB	80	Tan et al. (2011)
H. campisalis MCM B-1027	Maltose		56.23	Kshirsagar et al. (2013)
H. profundus	Carbon and fatty acids	PHB, PHB-co-HV	–	Simon-Colin et al. (2008)
Halomonas i4786	Saccharose and stachyose	PHB	78	Elain et al. (2016)
Bacillus sp.	Glucose	PHB, PHBV	–	Van-Thuoc et al. (2012)
Yangia pacifica	Glucose	PHB, PHBV	–	Van-Thuoc et al. (2012)
Bacillus megaterium H16	Glucose	PHBV	40	Berlanga et al. (2012)
Halophilic archaea				
Haloferax mediterranei	Agro-industrial residues	PHB-co-HV	60–65	Koller (2017)
Haloferax mediterranei	Glucose	PHBV	15.6	Melanie et al. (2018)
Halogranum amylolyticum	Unrelated carbon sources	PHBV		Zhao et al. (2015)
Halogeometricum borinquense strain E3	Sugarcane bagasse	PHB	45–50	Salgaonkar and Bragança (2017)
Natrinema pallidum 1KYS1	Corn starch	PHBV	53.14	Danis et al. (2015)
Natrialba strain 56	Acetate and *n*-butyric acid	PHB	53	Hezayen et al. (2000)
Natrinema ajinwuensis RM-G10	Glucose	PHB-co-HV	61	Mahansaria et al. (2018)
Haloferax mediterranei	Macroalgae hydrolysate	PHB-co-HV	57.9	Ghosh et al. (2019)

et al. 2008). A marine bacterial species *Halomonas* i4786 produced PHA utilizing reducing sugars from agro-industrial processing waters with 55% and 78% of PHA content of the biomass from leguminous processing water and fruit processing water, respectively (Elain et al. 2016). Acetic acid was used as a carbon source for enhanced production of PHA by halophilic bacteria (Wang et al. 2019).

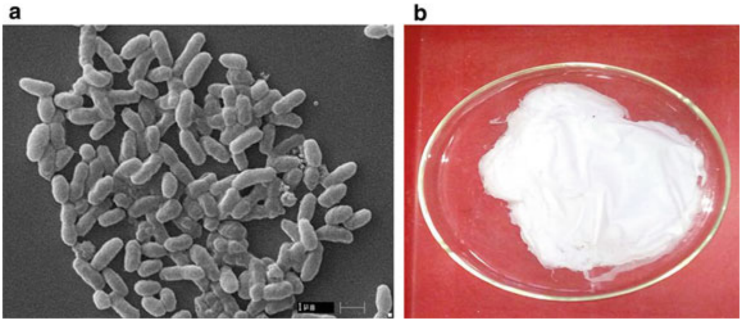

Fig. 10.1 PHB produced by *Halomonas venusta* KT832796. (**a**) SEM image of *Halomonas venusta* KT832796. (**b**) PHB film

Some of the halotolerant and halophilic bacteria isolated from mangrove soil belonging to *Bacillus* species and strains related to *Yangia pacifica* were capable of accumulating PHB and PHBV from glucose, thus indicating the capability of these microorganisms to accumulate PHA from diverse environmental niches (Van-Thuoc et al. 2012). Similarly, *Bacillus megaterium* H16, a moderate halophilic bacterium isolated from solar salterns, was found to accumulate PHA of 40% of the dry cell weight from 2% glucose with/without NaCl (Salgaonkar et al. 2013b). In another study, PHA accumulation by *H. alkaliphila*, *H. neptunia*, and *H. venusta* MAT-28 isolated from microbial mats were up to 40–60% of their dry weight (Berlanga et al. 2012). It was observed that *H. venusta* MAT-28 accumulated PHA as a stress-protective mechanism during the biofilm formation of culture growth (Berlanga et al. 2014). Hence, PHA accumulation indicates a response to the physiological adaptation in such an environment.

10.3.2 PHA Production by Archaea

PHA accumulation was first observed in the *Archaea Halobacterium marismortui* during characterization by a freeze-fracture technique that was isolated from the Dead Sea by Kirk and Ginzburg (1972) and Fernandez-Castillo et al. (1986). However, the PHA accumulation is usually not determined during characterization in haloarchaeal species. Based on the taxonomic studies, species of the family *Halobacteriaceae* that are able to synthesize PHA are *Haloferax*, *Haloarcula*, *Natrialba*, *Haloterrigena*, *Halococcus*, *Haloquadratum*, *Halorubrum*, *Halorhabdus*, *Haloferax sulfurifontis*, *Halobacterium*, *Haloterrigena*, *Halopiger*, *Halobiforma*, *Natronobacterium*, *Natronococcus*, and *Natrinema altunense* (Quillaguamán et al. 2010; Poli et al. 2011; Yogesh et al. 2012; Saharan et al. 2014).

The haloarchaeal species are also capable of producing PHB and copolymers using cheaper raw substrates from various agro-industrial wastes as a cost-effective process. The widely studied archaea for PHA accumulation is *Haloferax mediterranei* that has shown to utilize various agro-wastes as carbon sources. *Haloferax mediterranei* produced PHB-co-HV copolymers up to 60–65% of the biomass utilizing agro-industrial residues efficiently (Koller 2017). In another study, *H. mediterranei* produced PHBV of 15.6% of dry cell weight using glucose with phosphorus limitation condition (Melanie et al. 2018). Thus, culture conditions with varying nutrient supplies can seriously affect the PHA accumulation capacity of the microbial strain. A study showed that using non-conventional carbon sources such as macroalgae hydrolysate, *H. mediterranei* accumulated 57.9% (w/w) of PHB-co-HV to achieve sustainable alternatives for large-scale production of biopolymers.

Another haloarchaea *Natrinema pallidum* 1KYS1 was found to accumulate maximum PHBV up to 53.14% (w/w) utilizing corn starch, among other cheap carbon sources used, including sucrose, whey, apple, melon, and tomato wastes (Danis et al. 2015). PHA production was enhanced in *Natrinema ajinwuensis* RM-G10 that accumulated PHB-co-HV at 61% (w/w) by repeated batch culture process provided with glucose as the best carbon source after medium optimization (Mahansaria et al. 2018). Though haloarchaea are able to metabolize other carbon sources, they prefer glucose for maximum PHA accumulation. The PHBV copolymer synthesized from glucose as a sole carbon source is reported in *Halogranum amylolyticum* TNN58, with the 3HV fraction of 20.1% (w/w) being the highest reported so far by wild-type strains grown on unrelated carbon sources (Zhao et al. 2015). In another study, sugarcane bagasse was used to achieve maximum PHA of 45–50% (w/w) by *Halogeometricum borinquense* strain E3 compared to *Haloferax volcanii* strain BBK2, *Halococcus salifodinae* strain BK6, and *Haloarcula japonica* strain BS2 (Salgaonkar and Bragança 2017).

10.4 Fermentation Strategy for PHA Production: A Case Study of *Halomonas* sp.

Production of PHA in bioreactors under optimal conditions to obtain higher yields and productivities is an imminent step toward establishing commercial process. Toward this, several studies have been carried out using relevant microbial strains by elucidating the process strategies for efficient PHA production (Gahlawat 2019). However, this section is focused on process strategies for the production of PHA by *Halomonas* spp. Some of the upstream strategies for *Halomonas* spp. are consolidated in Table 10.2.

10.4.1 Optimization of Growth Medium

Optimizing the nutritional requirements for the production of a specific metabolite is the key to any bioprocess. Usually, for wild-type microbes, the nutritional requirements for growth and cell-derived biomolecule are different. Hence, there is a need to optimize the medium to achieve a trade-off between the cells and the

Table 10.2 Various process strategies for efficient PHA production

Strain	Mode of cultivation	PHA type	Strategy	References
Halomonas venusta KT832796	Fed-batch	P(3HB)	Single-pulse carbon (glucose) feeding	Stanley et al. (2018)
Halomonas venusta DSM4743	Fed-batch	P(3HB)	Initial glucose of 80 g/L and two-phase system was implemented	Gao and Zhang (2014)
Halomonas boliviensis	Fed-batch	P(3HB)	Addition of 0.4% (w/v) NH_4Cl and 0.22% (w/v) K_2HPO_4 along with intermittent addition of MSG to reactor	Quillaguamán et al. (2008)
Halomonas bluephagenesis TD01	Fed-batch	P (3HB-co-4HB), P (3HB)	Waste gluconate and CSL were used to scale up the production of P(3HB-co-4HB) in unsterile process	Ye et al. (2018), Ren et al. (2018)
Halomonas sp. KM-1	Fed-batch	(R)-3-HB	Used 200 g/L glucose as an initial concentration during aerobic batch cultivation followed by a shift to microaerophilic in fed-batch mode	Kawata et al. (2014)
Halomonas i4786	Batch	PHA	Leguminous processing water (LPW) and a mixture of glucose and fructose in fruit processing water (FPW) were used for PHA production	Elain et al. (2016)
Halomonas smyrnensis AAD6^T	Batch	P(3HB)	PHB co-produced with levan	Tohme et al. (2018)
Halomonas venusta MAT-28	Batch		PHA accumulation is found higher in immobilized cells detached from biofilm	Berlanga et al. (2012)
Halomonas boliviensis	Fed-batch	P(3HB)	Nitrogen limitation with low O_2 supply	García-Torreiro et al. (2016b)

product of interest. *Halomonas* spp. can utilize carbohydrates, amino acids, polyols, and hydrocarbons as sole carbon and energy sources (Kim et al. 2013). They prefer to grow in the range of 3–15% (w/v) of saline, being halophilic or halotolerant in nature. Phenotypic characteristics of 21 different types of strains of the genus *Halomonas* are provided in Mata et al. (2002), and specific differences among species on the capacity to metabolize certain compounds are detailed. Except *H. halodurans*, other 21 types of strains were capable of accumulating PHB (Mata et al. 2002). A defined medium is always preferable over complex medium, as the former has to control the growth and PHB production with respect to the individual nutritional component. Quillaguamán et al. (2008) observed that the optimization of the medium with respect to nitrogen source, phosphate, and dissolved oxygen resulted in improved PHB yield of *Halomonas boliviensis*. They have also observed that among all the amino acids, the addition of only glutamine, aspartic acid, and glycine could improve the dry cell weight (DCW) during batch cultivation. Glutamine led to the highest cell growth, and it was suggested that due to low solubility and high costs, glutamine could be substituted with MSG without any negative affect. As observed in another genus (Choi et al. 1998), nitrogen and phosphorus limitation can improve the volumetric productivities of PHB. The carbon-to-nitrogen (C/N) ratio was also observed to have an effect on biomass and PHA (Stanley et al. 2018). One of the key drawbacks of microbial PHA production is the cost competitiveness when compared to plastics from fossil fuels. The growth medium costs around 50% of the production expenses. Sustainable, cheap sources such as agricultural, municipal, industrial, and food-based wastes could be an alternative to a defined medium (Li and Wilkins 2020). With the addition of butyric acid and sodium acetate to wheat bran hydrolysate, they could increase the PHB titer to 4 g/L. In the study by Kovalcik et al. (2018), spent coffee ground (SCG) hydrolysate rich in carbohydrates (mannose, galactose, and arabinose) was used for accumulating PHA using *Halomonas halophila* CCM 3662. In their study, dried SCG was defatted, and phenolics were adsorbed onto XAD4 and finally acid/alkali-treated to obtain SCG hydrolysate rich in sugars, which was later used to obtain PHB titers of 0.95 g/L with a yield of 27% (w/w) of dry cell mass. In another study by Elain et al. (2016), leguminous processing water (LPW) and fruit processing water (FPW) were used for the cultivation of *Halomonas* i4786 to obtain PHA titers of 1.6 g/L and 1.8 g/L, respectively. However, from the above studies, it can be observed that using wastes as a carbon source results in lower yields and titers when compared to the defined medium. However, from an economic standpoint, optimizing the processes for the utilization of sustainable, cheap sources could result in the concept of biorefinery and bioeconomy.

10.4.2 Bioreactor-Scale Operation

Most of the halomonads are mesophilic in nature and grow optimally at 37 °C and neutral pH. The mode of cultivating the organism affects the product's titer and productivity. Usually, the batch cultivation cannot provide high titers as the initial substrate concentration is restricted due to substrate inhibition on the growth of the organism. A fed-batch strategy wherein the substrate is added to the reactor at a specific rate from a reservoir containing a high concentration of substrate not only improves the titers but also the productivity of PHB. From Table 10.2 content, it can be predicted that most of the studies exploited the fed-batch mode of operation to achieve high titers of PHB. The feed regime during fed-batch plays a major role in maintaining the physiology of cells to increase the rate of the product of synthesis. Here, the fed-batch can be operated either using feedforward (open-loop) or feedback (closed-loop) strategies. In the case of feedforward-based feeding, the dosage profile is predetermined based on material balance equations or certain heuristics, e.g., exponential feeding, constant feeding, linear feeding, etc. (Mutturi et al. 2017). In the case of feedback-based feeding strategies, a physiological parameter, such as pH, dissolved oxygen (DO), respiratory quotient (RQ), residual carbon, etc., is used as a closed-loop parameter to manipulate the feeding rate of the peristaltic pump. As mentioned earlier, several studies have reported that PHA accumulation occurs during nitrogen limitation. Hence, the feed reservoir usually is devoid of any nitrogen source. In the case of *Halomonas boliviensis*, the feeding was switched from glucose to volatile fatty acids to achieve nitrogen limitation and thereby increase the PHA production (García-Torreiro et al. 2016a). García-Torreiro et al. (2016b) observed that increasing KLa (volumetric mass transfer quotient) increased the biomass concentration in batch (shake-flask) experiments of *Halomonas boliviensis*. Based on this observation, the influence of oxygen during bioreactor experiments was carried to find the limitation of nitrogen, and low O_2 supply produced 35 g/L of PHB. It is reported that halophilic bacteria, which can withstand high osmolality, have the ability to grow at high initial carbon concentration (Quillaguamán et al. 2010; Kawata et al. 2014). Therefore, in the studies by Kawata et al. (2014), initial glucose for batch cultivation was set to 200 g/L, and later the aerobic cultivation was shifted to microaerobic over a period of 60 h in fed-batch cultivation by feeding sodium nitrate to obtain 40.3 g/L of (R)-3-HB. Similarly, different feedback-controlled feeding strategies were studied for PHA accumulation in *Halomonas venusta* (Stanley et al. 2018). They have noticed a maximum titer of 33.4 g/L when single-pulse glucose was added, which increased the residual sugar concentration to above 120 g/L. These two studies indicate that the *Halomonas* strains can withstand high residual sugars without any substrate inhibition. Based on the strain of *Halomonas* and feeding regime, fed-batch cultivation could certainly yield higher titers of PHB when compared to batch cultivation.

10.4.3 Downstream Processes for Effective PHA Recovery

Another major fraction of the overall microbial production of PHA involves in downstream processing/recovery of this biopolymer from fermentation broth. The downstream processing begins with the separation of cells via centrifugation, filtration, and sedimentation (Li and Wilkins 2020). The intracellular PHA is released from the cells by physicochemical procedures such as heating, freezing, grinding in liquid nitrogen, the addition of salts, etc. Recovery and purification of PHA are usually not specific to microorganisms. However, it is reported that halophilic bacteria can easily be subjected to hypo-osmotic shock with salt-deficient water (Quillaguamán et al. 2010). Kourmentza et al. (2017) detailed various steps in recovering the PHA after harvesting the biomass. In brief, biomass harvesting is followed by pretreatment, which could be lyophilization, thermal drying, and physicochemical or biological treatments. After this, PHA is extracted using solvents (halogen or non-halogen) or techniques such as supercritical fluids or aqueous two-phase systems followed by precipitation, polishing, and drying.

Pretreatment with 8 g/L of sodium chloride (NaCl) at 30 °C for 3 h has improved recovery to 97.5% in the studies by Anis et al. (2013). Thermal pretreatment, coupled with enzymatic digestion and chloroform extraction, has resulted in 94.1% recovery in the studies by Neves and Müller (2012). Other chemical disruption procedures include the utilization of sodium hydroxide (NaOH), sodium hypochlorite (NaClO), and sodium dodecyl sulfate (SDS). Almost 100% recovery was obtained using 5% NaClO when cells were subjected to disruption for 24 h in the studies by Villano et al. (2014), whereas 98% was recovered from *Halomonas* sp. SK5 when subjected to 0.1% SDS at 60 °C for 2 h or using distilled water at 30 °C for 18 h (Rathi et al. 2013). Kshirsagar et al. (2013) have carried out several recovery procedures for PHA from *Halomonas campisalis* involving sodium hypochlorite, chloroform, and SDS. They observed that 62% of the PHA was recovered when chloroform-based extraction was carried out using the Soxhlet apparatus. However, with the advent of osmotic lysis, ionic liquids, and supercritical fluid extraction, some advances are made in the downstream processing of PHA recovery. There is a need to optimize the downstream processes, as it is crucial to cut down the process costs significantly. Therefore, newer methods are constantly pursued to achieve higher recovery percentages using eco-friendly cheap process steps.

10.4.4 Metabolic Engineering of Halophiles for PHA Production

Advances in PHA production are being made in order to make PHA production an economically viable process. These involve a series of optimization strategies in halophilic and non-halophilic microorganisms such as supplying renewable carbon substrates, developing better bacterial strains, optimization of fermentation processes, engineering new pathways, etc. (Liu et al. 2015). There are reports on recent technology developed for genetic manipulation of halophilic bacteria such as gene overexpression, recombineering, gene knockout to achieve accelerated cell growth, and increased cell size for the accumulation of PHA granules (Fu et al. 2014; Zhao et al. 2017).

PHA production in recombinant *Halomonas* spp. is constructed by developing suitable molecular engineering tools (Tan et al. 2014). The studies on metabolic engineering in halophiles have led to the accumulation of PHBV copolymers with a high substrate to PHA conversion efficiency in *Halogranum amylolyticum* (Zhao et al. 2015) and *Halomonas bluephagenesis* (Chen et al. 2019). Among extremophiles, the halophiles are considered to be the future prospects in next-generation industrial biotechnology (NGIB) for PHA production, as reported by Chen and Jiang (2017). A recent study reported the production of P(3-HB) and (P-3HB-co-3HV) by *Halomonas* sp. SF2003, and the functionality and differences between its two PHA synthase genes (*pha*C1 and *pha*C2) were tested in a non-PHA-producing transformant strain (Thomas et al. 2020).

10.5 Applications of PHA

The PHB and its copolymers have wide applications in medical, agricultural, and packaging industry (Vijayendra and Shamala 2014). They are used as nanocomposites, and chiral monomers in pharmaceuticals and novel applications are being developed in recent years. However, PHA-based materials could not reach the common man due to its high cost compared to petroleum-based non-biodegradable polymers. Chen et al. (2020) very recently have reviewed various reasons and solutions for this issue. Some of these include high-energy consumption, unstable PHA polymer and its molecular weight, expensive downstream processes, difficulty in achieving high cell density, and its hard cell wall. Some companies are using PHA for the production of disposable items replacing conventional plastics. PHA was used to produce shampoo bottles by BioPol Company in the UK. It is used to produce water-resistant surfaces covering over cardboard and in making foils, films, and diaphragms. Biomer, a German company, uses PHA to make combs, pens, and bullets. PHA blend of P(3HB) and poly(3-hydroxyoctanoate) made by Metabolix, a US company, is used to produce food additives and it is approved by the FDA. P(3HB) and its copolymers are used to make flushables, nonwovens, binders, flexible packaging materials, thermoformed articles, synthetic paper, and articles by the collaborated companies of the USA, China, and Korea using P (3HB-HHx). P(3HB-HV) is used in making coated paper milk cartons. Nodax, a PHA-based company of Procter & Gamble, uses P(3HB) with mcl-monomers [P (3HB-3HV)] as coatings of urea fertilizers, herbicides, and insecticides.

Owing to their piezoelectric nature, PHA granules are used in making pressure sensors, like keyboards and measuring instruments, in material testing, as shock wave sensors, and in gas lighters. They are used in acoustics like microphones, ultrasonic detectors, sound pressure measuring instruments, oscillators, headphones, and loudspeakers, in ultrasonic therapy, and in atomization of liquids, respectively. In medical applications, P(3HB) and its copolymers, viz., P(3HB-3HV), P(4HB), P (3HO), and P(3HB-3HHx), are mainly used for making bone plates, osteosynthetic materials, and surgical sutures. They can also be used in the form of capsules for slow-releasing drugs and hormones, swabs and dressing materials used for surgery, cardiological stents, heart valves, vascular grafts, and artery augments, in tissue engineering, etc. (Philip et al. 2007; Keshavarz and Roy 2010; Gumel et al. 2013; Ali and Jamil 2016). Production of functional PHA molecules with specific properties, (like PHA organo-hydrogel, with enhanced hydrophilicity and thermoresponsibility, superhydrophobicity, besides, other applications in the biomedical field are contemplated by several researchers, and it was reviewed very recently (Chen et al. 2020).

10.6 Conclusion

The study on halophilic microorganisms for sustainable production of PHA at the industrial scale is an important step to curb the high production cost. Several aspects of the PHA production, such as cheaper carbon substrates from agro-industrial wastes as raw materials, coupled with the halophilic bacteria's metabolic ability to utilize it and to accumulate PHA and its copolymers are useful steps. However, using halophilic microorganisms at an industrial scale has certain challenges. The high salt concentration affects the walls of the bioreactor vessel leading to corrosion. Hence, to overcome this, use of saline-alkali bioreactors such as plastics, ceramics, or carbon steels can be replaced instead of the stainless steel fermentors and piping systems. In addition, the saline wastewaters can be recycled or treated using marine bacteria, which help in waste treatment management and cost reduction.

Nevertheless, the robustness of halophiles has an overall advantage for using them as potential candidates for bioproduction of PHA at the industrial level as they thrive in such harsh conditions and its easy maintenance. Thus, they serve as a potential competing group of microorganisms to produce PHA at a better biotechnological level. With the progress in genetic and metabolic engineering of the PHA pathways and the recombinant halophilic producers, there is a possible improvement for PHA and copolymer production, besides easy downstream processing. Application of PHA and copolymers with suitable characteristics can be further tuned for desirable properties. It further enhances its product value toward the sustainable development of biopolymer production.

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Aliphatic Biopolymers as a Sustainable Green Alternative to Traditional Petrochemical-Based Plastics

11

Rafeya Sohail and Nazia Jamil

Abstract

The use of traditional petrochemical-based plastics has led to increased fossil fuel utilization, CO_2 emissions, and plastic waste generation. Sustainable, renewable, biocompatible, and largely biodegradable bioplastics especially aliphatic biopolymers such as polyhydroxyalkanoates and polylactides show high potential as environment-friendly, green alternatives to traditional plastics. Bioplastics have a relatively shorter residence time and unfavorable impact on environment. Often, these biopolymers negate the long-term environmental effects and negative carbon footprint caused through landfill and incineration of petrochemical-based plastics. Copolymer blends of these biopolymers serve to relieve many obstacles encountered during product development, sustainability, and degradation in research and industry. The demand of the day is to highlight types, process development challenges, production statistics, sustainability, degradation, and applications of such biopolymers. The present chapter focuses on the nature, material characteristics, productivity, and potential of aliphatic biobased and bioderived polymers along with their current or emerging applications.

Keywords

Aliphatic biopolymers · Copolymerization · Degradation · Polyhydroxyalkanoates · Polylactides

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11.1 Introduction

Traditional plastics based on fossil fuels and petrochemical have been an integral part of our daily life since the first plastic production in the 1940s (Johnston et al. 2017; Radecka et al. 2016). Since then, the use of plastics has only increased with the advent of research outlining their properties, i.e., lightweight, durability, strength, elasticity, and especially the easily achieved physical and chemical manipulation befitting current purpose (Reddy et al. 2003). On the other hand, this excessive use of plastics has, by default, also increased the plastic waste percentage. Approximately 14% of the total generated waste in China is comprised of plastics. In Europe and the USA, 7% and 11.8% of total solid waste is due to plastics, respectively (Radecka et al. 2016). Impediments met during disposal, recycling, and degradation of plastics have led to environmental pollution crisis (Verma et al. 2016). Even degraded plastic fragments—of less than 5 mm—are not actually disposed of completely. Several reports detail their ingestion by sea life and infiltration into the food chain (Johnston et al. 2017). Recycling and disposal of plastics also raised many issues, such as deplition of landfills and, dumping in oceans, etc. (Fossi et al. 2020). Around 60–80% of all debris in waterbodies accounts for plastic waste (Johnston et al. 2017).

In view of all this, the need for sustainable bioplastics has increased now more than ever (Muniyasamy et al. 2019; Radecka et al. 2016). This interest stems not only from concerns related to limited supply of fossil fuel but also from those related to eventual disposal of synthetic plastics (Zhang and Thomas 2011). Biodegradable plastics are easily recyclable and biocompatible and occupy much less time in environment after disposal (Kubowicz and Booth 2017). Biobased plastics also exhibit non-toxic, thermoplastic, and elastomeric properties (Vijay and Tarika 2019). The main problem faced in commercialization of biodegradable plastics is high production cost. There is a need to optimise low cost economical methodologies (Leja and Lewandowicz 2010).

There are three groups of natural biodegradable polymers (Reddy et al. 2013). In the first group, there are mixtures of easily digestible substances, i.e., starch, cellulose, etc., and their derived polymers (Fabunmi et al. 2007). The second group consists of synthetic polymers made up of chemical complexes susceptible to microbial digestion by hydrolysis. The last and most popular group contains biobased aliphatic polyester obtained from bacterial sources (Sohail et al. 2020; Sohail and Jamil 2019) (Fig. 11.1). Few important biodegradable polyesters include polyhydroxyalkanoates, poly(ε-caprolactone), polylactides, polyglycolic acids, polyethylene oxide, cellulose-based plastics, lignin-based polymer composites, starch blends, thermoplastic proteins, etc. (Rogovina 2016). Many microbes such as *Bacillus*, *Pseudomonas*, *Actinomycetes*, *Mycobacterium*, *Rhodococcus*, *Micromonospora*, *Flavobacterium*, *Azotobacter*, *Comamonas*, *Alcaligenes*, *Escherichia*, *Streptomyces*, *Nocardia*, etc. are capable of producing and accumulating biopolymers in levels as high as up to 90% of total dry cell weight (Leja and Lewandowicz 2010). Among all these, polyhydroxyalkanoates and polylactides have extensive import due to their far-reaching applications. Blends

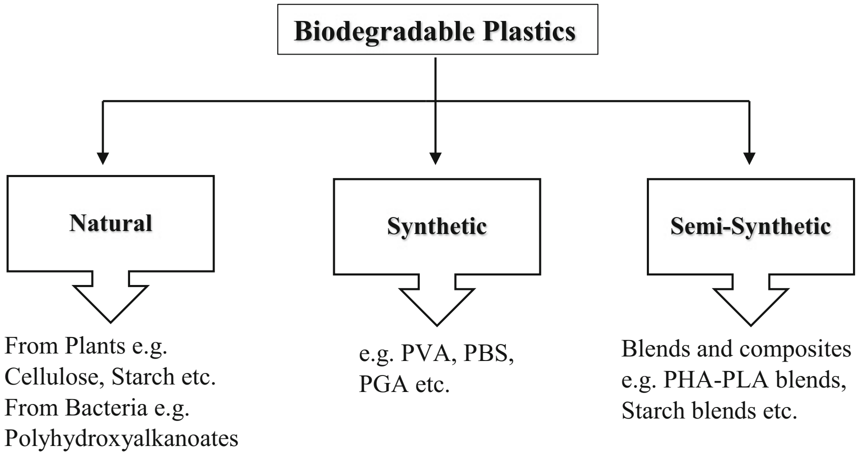

Fig. 11.1 Classification of biodegradable plastics

of polyhydroxyalkanoates and polylactides have been reported to show enhanced desirable properties and degradation rates. These biopolymers are being discussed below.

11.2 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are polyesters composed of various hydroxyalkanoates (Aljuraifani et al. 2019). These hydroxyalkanoates are synthesized by many bacteria under stress, as a reserve for carbon and energy (Li et al. 2016; Reddy et al. 2003). After biosynthesis, accumulated PHAs can either be processed directly for bioplastic generation or indirectly with compatible organic and/or inorganic materials to generate composites and blends or for conversion to functional follow-up compounds (Sohail et al. 2020; Sohail and Jamil 2019; Koller et al. 2015) (Fig. 11.2). Although this wide range of features provides PHA with a competitive edge over its non-degradable, petrochemical-based, synthetic plastic counterparts, the main property setting PHA apart from its petrochemical-based counterparts is the biodegradability of PHA upon marine sedimentation, exposure to soil, and composting (Radecka et al. 2016). PHAs serve as key components in regulation of cell motility, osmoregulation, and carbon routing during different metabolic processes (Tan et al. 2014; Koller et al. 2015). PHAs also aid in survival mechanisms by spore and cyst formation (Leja and Lewandowicz 2010; Foster et al. 2001).

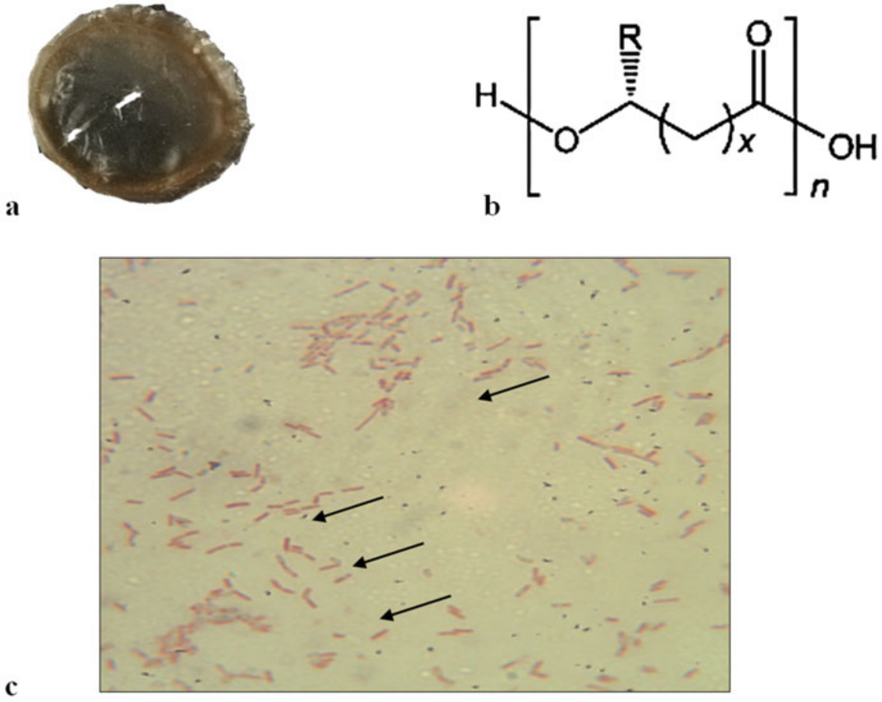

Fig. 11.2 (**a**) PHA film obtained through solvent extraction. (**b**) General molecular formula of PHAs. *X* ranges from 1 to 8, and *n* ranges from 100 to 1000 s. (**c**) Sudan staining micrograph of PHA producers depicting dark PHA granules inside pink-stained bacterial cells

11.2.1 Chemical Nature of PHA

To date, more than 150 different monomer units composing PHA polyesters have been identified and studied (Tan et al. 2014). These monomers control the overall elastic behavior, stiffness, crystallization kinetics, and degradation of PHA molecules (Koller 2018). Monomers are incorporated into PHA chains and form three distinct classes, i.e., short-chain-length PHA (scl-PHA), medium-chain-length PHA (mcl-PHA), and long-chain-length PHA (lcl-PHA). Short-chain-length PHAs are composed of monomers with three to five carbon atoms (3HV, 3HB, etc.). Medium-chain-length PHAs are composed of monomers with 6–14 carbon atoms (3HHx, 3HO, 3HD, 3HDD, etc.) (Sharma et al. 2017). Long-chain-length PHAs are composed of monomers with more than 16 carbon atoms (Tan et al. 2014). Properties of PHAs are strongly dependent on their monomeric composition and structures, i.e., poly(3-hydroxybutyrate-co-hydroxyvalerate) has lower crystallinity and melting point than poly(3-hydroxybutyrate), whereas polyethylene has low tensile strength and malleability as compared to poly(4-hydroxybutyrate) (Povolo et al. 2013). It has been reported that specific monomer incorporation enhances thermal stability, i.e., an increase in poly(3-hydroxybutyrate) concentration among PHA monomers increases melting point (Khosravi-Darani et al. 2013).

11.2.2 Biosynthesis of PHA

The variance in concentration level of PHA formation, nature of polymerization, and substrate diversity across different species depends on the biosynthesis pathway followed (Aljuraifani et al. 2019). Synthases, phasins, depolymerases, and other regulatory proteins involved in PHA synthesis and degradation are integrated in "carbonosomes"—a dense phospholipid layer (Ray and Kalia 2017). PHA synthase enzymes are encoded by numerous genes (Solaiman et al. 2000). Among these, genes *phaA*, *phaB*, *phaC*, and *phaJ* encode β-ketothiolase, acetoacetyl-coenzyme A reductase, PHA synthase, and enoyl-coenzyme A hydratase, respectively. Several other genes also affect PHA formation, i.e., gene *fabG* that encodes 3-ketoacyl-acyl carrier protein (ACP) reductase (Numata et al. 2013).

11.2.3 Applications

PHAs are effective substitutes for conventional synthetic and petrochemical-based plastics due to their biodegradability, biocompatibility, and low toxicity. Due to their similarity with their synthetic counterparts, PHAs have widespread application in bioplastic production (Aljuraifani et al. 2019; Koller et al. 2015). In therapeutics and pharmaceutical fields, PHAs have been approved by the US Food and Drug Administration (USFDA) for application as tissue engineering scaffolds, implants, repair patches, meshes, nerve repairs, artificial organs, and controlled drug release due to its non-toxicity and biocompatibility (Shah and Vasava 2019; Koller 2018). Micro- and nanospheres of PHAs are being used for anticancer therapy (Shah and Vasava 2019). In computer-assisted tomography and ultrasound imaging, PHAs are utilized as contrast agents (Shah and Vasava 2019). PHAs are utilized as raw material for bioactive material such as probiotics, pheromones, etc. (Ray and Kalia 2017). Studies are being conducted to manipulate the structure of PHAs in order to enhance their thermal properties as well as crystallization kinetics (Gopi et al. 2018). Genome editing and various synthetic biology tools are being used to improve metabolic engineering of PHA (Zhang et al. 2020). Studies to increase yield of PHA production using transgenic crops are being conducted. Volatile fatty acids are being used for reduction of production costs (Wang et al. 2019).

11.3 Polylactides

Polylactides (PLA) are aliphatic polyesters whose biobased production from natural or naturally derived sources such as corn has been well documented (Shah and Vasava 2019). Due to its biodegradability, low processing temperature, excellent renewability, high modulus, easy moldability, and biocompatibility, it has widespread utilization in biomedical applications as well as in commodity (Msuya et al. 2017; Ausejo et al. 2018). PLA has also been USFDA approved for clinical applications (Shah and Vasava 2019). PLA polyesters taking only few months or years at most for complete degradation serve as a green alternative to petrochemical-based plastics taking more than 500 or 1000 years (Msuya et al. 2017).

11.3.1 Chemical Nature

These biopolymers have been grouped α-hydroxyl acids derived polyester family (Msuya et al. 2017). Polylactides have four different stereoisomeric forms based on these enantiomeric stereocenters, i.e., L-,d-; L,L- and -d,L- (Kricheldorf 2001), which control the crystallizable content of PLA (Zhang and Thomas 2011). These stereoforms of PLA include poly-L-lactide (PLLA), poly-D-lactide (PDLA), poly-D,L-lactide (PDLLA), and poly-L,L-lactide (PL-LA) (Msuya et al. 2017; Pretula et al. 2016). The poly-L-lactide (PLLA) and poly-D-lactide (PDLA) forms can be blended to form stereo-complex PLA with melting temperature ranging from 220 to 230 °C (Msuya et al. 2017). PLA molecules are held together by polar oxygen linkages which are responsible for its hydrophilicity. The hydrophobicity of PLA polymer, however, is imparted by methyl group side chain (Shah and Vasava 2019). Based on the polymerization process, there are two types of polylactides. Direct polycondensation of lactides forms low molecular weight polylactides, while ring-open polymerization (ROP) of lactides gives rise to cyclic diesters of lactic acid and forms high molecular weight polylactides (Pretula et al. 2016).

11.3.2 Physical Nature

The physical nature of PLA depends on the monomeric stereoregularity of its polymeric backbone (Shah and Vasava 2019; Su et al. 2019). These polyesters are rigid having thermal and mechanical properties that are comparable to polyethylene terephthalate (Zhang and Thomas 2011).

Physical properties such as crystallinity and melting temperature of PHA are wholly dependent on its chemical configuration, i.e., PLLA and PDLA both exhibit different properties (Su et al. 2019; Msuya et al. 2017). As PLLA show a high melting temperature, i.e., 173–178 °C, and glass transition temperature, i.e., 55–80 °C, while PDLA has a comparatively low melting temperature, i.e., 120–150 °C, and glass transition temperature, i.e., 40–50 °C (Shah and Vasava 2019). Both PLLA and PDLA are semi-crystalline. These exhibit no or very low toxicity and have high mechanical performance compared to synthetic polymers (Leja and Lewandowicz 2010). The solubility of PLA is also dependent on its enantiomeric stereocenters, i.e., PLLA is soluble in chloroform, dioxane, dioxolane, furan, etc., while PDLA is soluble in THF, DMSO, ethyl acetate, ethyl lactate, DMF, and *N*,*N*-xylene (Shah and Vasava 2019).

11.3.3 Synthesis of Polylactides

Polylactide can be synthesized via various processes such as ROP of lactide (dimer of lactic acids known as lactone ring), azeotropic dehydration, polycondensation, enzymatic polymerization, etc. (Shah and Vasava 2019). The cheapest method among all these is polycondensation, which yields low to high molecular weight PLA. This PLA can be converted to high molecular weight PLA either by use of esterification adjuvant, e.g., diisocyanates, or chain-extending agents, e.g., dicarboxylic acids. However, ROP and azeotropic dehydration are employed to achieve high molecular weight PLA (Kim et al. 2006). During these synthetic processes—in order to manipulate polymerization equilibrium—water molecules are removed with the help of azeotropic solvents which, in turn, aids in production of high molecular weight PLA at a temperature lower than its melting temperature (Shah and Vasava 2019).

PLA is generally synthesized either by direct polycondensation or ring-opening polymerization of either lactides or L-lactic acid monomers (Pretula et al. 2016). There are two key steps in production of polylactides by either direct polymerization or ring-open polymerization. The first step is the conversion of glucose to lactic acid, while the second step is the conversion of lactic acid to polylactide or polylactic acid (Keziah et al. 2018). However, both processes include the use of poisonous solvents that deviate from biopolymer and create a paradox with green chemistry principles. Several methods are under study in order to synthesize PLA based on green chemistry principles (Muller et al. 2017). These methods include the use of cheap, unharmful natural resources for PLA production (Rudnik 2019; Royte 2006). Studies have also been conducted on the use of staple food items for bioplastic production (Kothekar et al. 2018).

11.3.4 Applications

In medical field, PLA are used as ligament replacements, artificial grafts, sutures, and orthopedic screws and in controlled drug release (Muniyasamy et al. 2019). In therapeutics, PLA are USFDA approved for use as tissue cultures, wound closure, surgical implants, prosthetic devices, resorbable surgical sutures, and controlled release systems (Shah and Vasava 2019). In industrial packaging PLA was used as food packaging polymer. (Msuya et al. 2017; Gadhave et al. 2018). Products of PLA have been modified to increase crystallization kinetics by blending, chemical modification, and addition of plasticizing or nucleating agents, etc. (Shah and Vasava 2019).

11.4 Copolymerization of Polyhydroxyalkanoate and Polylactide Copolymers

PHA are highly elastic and have high crystallinity, low viscosity, and thermal stability but comparatively low biodegradability (Su et al. 2019). PLA, on the other hand, are rigid, have low elasticity, and exhibit low heat distortion at high temperatures. PLA also show comparatively high biodegradability and poor and slow crystallization rate. Increased elasticity, biodegradability, crystallinity, and thermal stability have been reported for PHA-PLA blends (Roy and Visakh 2014). However, PHA are miscible only with low molecular weight PLA. In the case of high molecular weight PLA, PHA-PLA blends are prepared by compounding, hot pressing, etc. (Zhang and Thomas 2011). However, the thermodynamically favored biphasic composition of PHA-PLA blends—regardless of their specific properties—often leads to restrictions on their applications (Su et al. 2019). Additionally, in the case of PHB, there is narrow "window of processability" due to the low difference between its melting point (~180 °C) and decomposition temperature (~270 °C) (Koller et al. 2015). After overcoming these obstacles, copolyesters with desirable enhanced properties and application can be made.

11.5 Biodegradation of PHA, PLA, and PHA-PLA Copolymers

Biodegradability of PHA, PLA, and PHA-PLA copolymers is dependent on environmental conditions, i.e., pH, temperature, oxygen, water content, presence or absence of microorganisms, etc. Many microbial enzymes cannot degrade biopolymers with C-C backbone easily (Su et al. 2019). In polymers having backbone composed of degradable chains, biodegradation is influenced by the structure of polymer on nano- and/or microscale, i.e., crystallinity, morphology of blends, and orientation. There are two main mechanisms in hydrolytic degradation, namely, non-enzymatic mechanisms and enzymatic chain scissions. During enzymatic chain scission, polymer's surface regions are degraded, while during non-enzymatic degradation, low molecular weight degradation products are disintegrated via dissolution or taken up by microbes adhering to the polymer's surface (Su et al. 2019). Additionally, surface degradation of amorphous regions progresses more easily than that of crystalline regions.

Microbial enzymes break down PHA polymers to basic building blocks, namely, hydroxy acids, and utilize these as carbon source. These hydroxy acids also serve as chiral building blocks for biochemical production (Johnson et al. 2009). Abiotically, PHA degradation occurs by hydrolyzation of ester bonds without catalysis or any enzymatic action. The residual products of PHA degradation are biodegraded (Shah and Vasava 2019) by extracellular (ePhaZ) and intracellular depolymerases (iPhaZ) to oligomers and monomers (Ray and Kalia 2017). Intracellular depolymerases are reported in *Alcaligenes faecalis*, *Pseudomonas fluorescens*, *Rhodospirillum rubrum*, *Comamonas acidovorans*, *Ralstonia eutropha*, etc. as transmembrane proteins. Depolymerases acting extracellularly on PHA are substrate specific, i.e., scl-PHA depolymerases and mcl-PHA depolymerases (Ray and Kalia 2017). Hydrolytic degradation of PLA is dependent on the properties of PLA polymer as well as the pH and temperature. Polymer properties include factors such as hydrophobicity, hydrophilicity, molecular structure, crystallinity, polydispersity index, chemical stability, presence of trace amounts of additives, softening agents, and pollutants. PLA decomposes, within 6–12 months, under humid conditions depending on its nature, chemical composition, and chirality either by bulk erosion or surface erosion. Degradation starts with molecular weight reduction due to hydrolysis followed by fragmentation of PLA into lactic acid, oligomers, and water-soluble by-products by random ester bond cleavage. These products and by-products are utilized by microbes and degraded completely into CO_2, water, and biomass (Shah and Vasava 2019). Environmental biodegradation of aliphatic polyester copolymers occurs in three main steps. Firstly, there is no or little weight loss or weight gain in case polyester is hydrophilic in nature. Secondly, random chain splitting causes reduction in weight and mechanical properties, i.e., tensile strength and elongation. Lastly, fragmentation of polymers occurs as a result of morphological breakdown (Su et al. 2019). Biodegradable thermoplastics like PHA and PLA have great potential in almost all industries (Queirós et al. 2015; Iwata 2015).

11.6 Future Perspectives

The most significant setback faced in successful commercialization of biopolymers is the production costs. However, production costs—as high as 40–60%—have to be reduced to gain a competitive edge (Mahishi et al. 2003). To lower production costs, different plant-derived carbon sources, e.g., glycerol, bagasse, molasses, starches, etc., are being employed on lab scale as well as commercially (Cavalheiro et al. 2009). The eventual degradation products (carbon dioxide and water) of these bioplastics are—in turn—taken up by plants in another cycle (Fig. 11.3). Studies to lessen production costs by utilizing low-cost resources, downstream processing, use of agro-industrial wastes, and ozonolytic fatty acid degradation are being conducted (Aljuraifani et al. 2019; Koller et al. 2015).

Studies are also being conducted to overcome challenges faced in modification of PHA-PLA surface, since there is a lack of any side chain groups that are modifiable. Rasal et al. (2008) also used photografting approaches to modify surface properties of PLA and PHA.

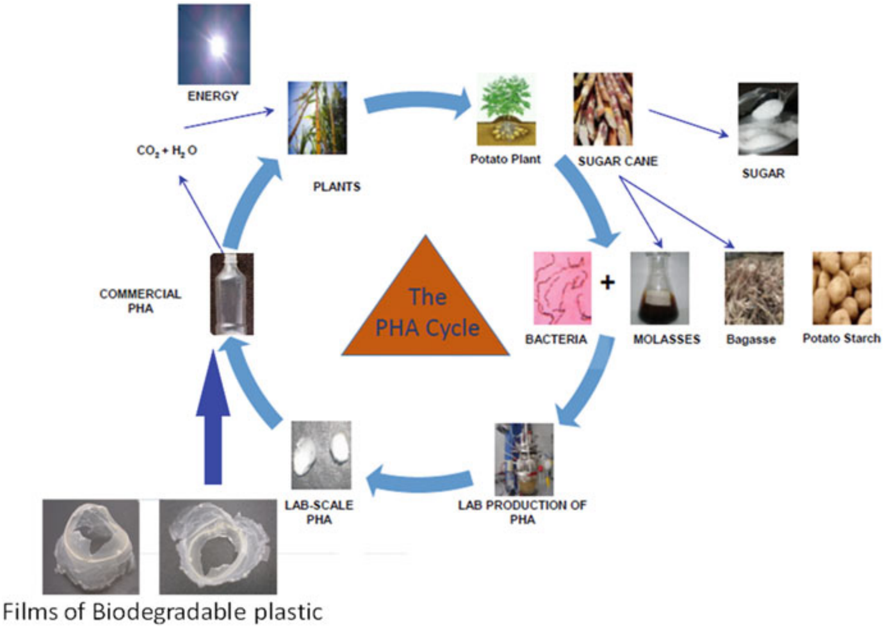

Fig. 11.3 Lab-scale and commercial PHA production: PHA films produced using plant-derived molasses, bagasse, and starches are utilized commercially as bioplastics that are eventually degraded completely to carbon dioxide and water

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Bioplastic: Food and Nutrition

12

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Abstract

Synthetic plastic has become an important part of modern lifestyle due to its broader range of applications in various sectors of life like packaging industries and food industries. As most of the synthetic plastic is obtained from petrochemicals, the disposal of this synthetic plastic now becomes one of the major problems of today's world. They persist in the environment in the form of improperly disposed of material which is a source of environmental pollution. Some of the major hazards of synthetic plastics are that they are carcinogenic, high source of carbon emission and harmful for marine life and decrease the soil fertility. To resolve these problems, the world is considering different alternatives to synthetic plastic to overcome this hazard. The biopolymer obtained from natural resources such as protein and polysaccharides is a sustainable source of bioplastic production as it is completely biodegradable and compostable. The biopolymers are eco-friendly compound and are used in a number of medical applications like surgical aids and coating of drugs and medicines. The biopolymers like starch and agar due to their nutritional value are also an important food source throughout the world. The biopolymers obtained from natural resources are non-toxic, non-carcinogenic and antimicrobial due to which they can be used in food packaging industries as well as in pharmaceutical industries. The use of bioplastics in food packaging increases the shelf life of the food and preserves it for a longer period as compared to synthetic plastics. It is observed that bioplastics are non-hazardous for livestock as they are completely naturally derived eco-friendly material.

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12.1 Introduction

12.1.1 Food and Nutrition

To keep your body healthy, you need to make smart food choices and protect your body from diseases and infections. Taking up food and nutrition serves as a body fuel, and we need to supply nutrients such as water, carbohydrates, fats, proteins, vitamins and minerals to our body to keep our body working. A healthy diet plays a key role in the physical and mental health of any individual, especially children. To fulfil the requirements, we must include a small portion of fruits, vegetables, wheat, rice, pulses, meat, seafood, eggs and nuts every day in our diet to overcome malnutrition. If an individual doesn't get the optimum amount of the required dietary requirement, then one can face chronic energy deficiencies. As per current situation, the world is facing major food security challenges despite the advancement and abundance in food production globally. There are millions of life forms that die annually due to hunger. There are continuous depletion of earth resources observed such as freshwater resources, degradation of land, infertility of soil, overfishing and global warming due to drastic climate changes (Tsang et al. 2019). In addition to that, in developing countries, an increase in human population is another major reason of not getting surplus amount of food, resulting in poorer communities. The scientists are continuously working on advancement in agricultural field and other renewable resources to produce surplus food in order to feed the entire population.

One of the foremost reasons of the shortage of food globally is its wastage during the processing of food at different stages (Tsang et al. 2019). It is estimated that approximately 1.3 billion tonnes of food including domestic, commercial, industrial and agronomic residues are wasted annually. The wastage of food is the wastage of all the other resources such as land, water and minerals which are utilized during production of food, resulting in further economic loss that leads to harmful environmental impact such as shortage of water, infertility of land, high carbon footprint, etc. (Baum et al. 2016; Tian et al. 2016). To keep the food safe, the synthetic packaging material was introduced which later became a major threat to the environment (Tsang et al. 2019). The synthetic plastics are highly carcinogenic and toxic in nature as they are derived from petroleum-based material. The synthetic plastics are also non-biodegradable and accumulated in the form of solid waste on earth (Hira et al. 2018). About 40% of these single-use food packaging materials end up in soil and ocean where it causes lethal effects on all life forms. Due to these synthetic plastic packaging, many of the marine species have also become endangered, and if the issue is not resolved by 2050, there will be more synthetic plastic in the oceans than marine organisms (Tian et al. 2016).

It is suggested that if the food waste and food packaging waste are properly converted into value-added products like bioplastic and biogas, respectively, we can save huge economic losses and energy crises (Suyasa and Dwijani 2015; Troella et al. 2006; Rajendran et al. 2012; Tian et al. 2016). The utilization of food waste in production of bioplastic is a sustainable and eco-friendly alternative, in which plastics are synthesized from renewable resources (Guillard et al. 2018). Food waste is also one of the main sources of landfills which produce greenhouse gas emission and groundwater contamination. However, when food waste is converted into bioplastics, it becomes economically valuable as it can be used in packaging and production of bio-based products, which can be easily decomposed without harming the environment. Moreover, the use of bioplastics in gardening pots increases the life span of the plant as it does not leave residues and increases soil fertility. Use of bioplastics in disposable crockery reduces land litters, and disposal of bioplastics in water does not harm marine life because it is soluble in water and acts as a water-purifying agent (Suyasa and Dwijani 2015; Troella et al. 2006; Rajendran et al. 2012).

12.1.2 Deteriorating World Resources Due to Plastic Pollution

The continuous changes in climatic conditions and depletion of fossil fuel resources, natural gas, and oil are an alarming situation for the world; that's why scientists are looking for more sustainable solutions to save the world resources. In addition to that, there is a global consumption of plastic because of its low cost and versatility. The plastic exhibits excellent flexibility, elasticity and moulding ability into a variety of shapes which have made it an important and reliable material for packaging industries as it can be used in the formation of various applications (Rajendran et al. 2012). The plastics are obtained from natural gas and petroleum-based polymer. The most used petrochemical-based plastics in packaging industries are polyethylene terephthalate (PET), polyvinylchloride (PVC), polyethylene (PE), polypropylene (PP), polystyrene (PS) and polyamide (PA). But these petrochemical-based plastics are non-recyclable and cause many of the economic and environmental imbalances in the world; that's why the scientists are searching for different bio-based polymer to produce bioplastic which is economically viable and competitive to petroleum-based plastic (Johansson et al. 2012; Rajendran et al. 2012; Gade et al. 2013; Sudesh and Iwata 2008).

12.2 Bioplastics

Bioplastics are the form of plastics which are obtained from partially or completely renewable biomass resources such as sugarcane, vegetable fats and oil, corn starch, pea starch, potato starch, cellulose, trees, seaweeds and microbiota. The term bioplastic represents a material which either possesses bio-based or biodegradable property or contains both properties (Arikan and Ozsoy 2015; Rajendran et al.

2012). According to the European Bioplastic Association, bioplastics can be categorized into three main groups (Johansson et al. 2012):

1. Bio-derived and biodegradable/compostable, e.g. polylactides and polyhydroxyalkanoates.
2. Fossil fuel-derived and biodegradable, e.g. polycaprolactone.
3. Bio-derived and non-biodegradable, e.g. bio-polyethylene (BIO-PE) and bio-polyethylene terephthalate (BIO-PET).

The term bio-based means the plastic is partly derived from natural resources such as starch, cellulose, chitin and polysaccharides (Chen 2014), whereas the term biodegradable is referred to the material that can be broken down into substances such as carbon dioxide, water and compost by microorganisms and bacteria. The biodegradability of material is mainly dependent upon environmental factors such as temperature, moisture, availability of oxygen and humidity (Rajendran et al. 2012). The biodegradability of any bioplastic is one of the important environmental aspects. The other important aspect is the compostability of bioplastic polymers so that they can act as fertilizer and soil conditioner without leaving any toxic effects. However, it is not necessary that the biopolymer obtained from sustainable resources must be decomposable. The biodegradability is mainly dependent on the biochemical structure of biopolymer rather than its source. The chemical bonding of the material defines the time taken by microbes to biodegrade the material. That's why many of the synthetic bioplastic polymers are biodegradable such as starch, lignin and cellulose. However, many natural polymers lose their biodegradability because of chemical modifications like polymerization (Guilbert et al. 1997; Petersen et al. 1999; Siracusa et al. 2008).

Plastics are compounds composed of different materials like polymers, plasticizers, stabilizers, colourants and additives. The plasticizer and other additives usually give mechanical strength to plastic such as elasticity and flexibility. The composition of the polymer and other materials keeps changing according to the type of the required product, its processing and future applications. However, manufacturing of a bio-based product still needs synthetic material for its stability. Hence, many of the bioplastics are yet to be mixed with synthetic components such as polymers and additives to improve their functionality and applications (Siracusa et al. 2008).

12.3 Biopolymer

The term biopolymer is used to define a polymer which is derived from renewable resources. The biopolymer includes both naturally occurring and synthetic bio-based polymers which are classified according to their chemical composition, method of processing and synthesis, their application and economic value (Khan et al. 2017). There are many biopolymers which are used for the packaging material because of their properties like high gas barrier property and flexibility. These biodegradable

Table 12.1 Classification of biopolymer

S. no.	Type of bioplastic polymer	Origin of extraction of polymer	Uses
1.	Natural polymer Polymer directly extracted from the natural biomass	Lipids, proteins and carbohydrate	Used in coating of fruits, nuts and frozen food To produce micro−/nanostructure suitable for medical application Medicine and novel drug coating
2.	Biochemosynthetic polymer Polymer produced by 'classical' chemical synthesis using bio-based derived monomers	Polylactide (PLA) and its derivatives	Used in biomedical applications in the area of surgical sutures, controlled drug release and others
3.	Biosynthetic polymer Polymer produced by genetically modified bacteria or microorganism	Polyhydroxyalkanoates (PHA) and its derivatives	Treatment of wastewater; development of implanted medical devices for dental, orthopaedic and skin surgery; medical devices; and drug delivery system

and eco-friendly polymers may be classified into three groups based on their origin of extraction and synthesis as shown in Table 12.1.

As mentioned earlier, not all biopolymers are biodegradable nor edible, e.g. crystalline polylactic acid (PLA) is a non-biodegradable bio-based polymer. Similarly, the starch-based plastic needs some chemical treatment to remove the hydroxyl group to ester or ether group. Other modifications include cross-linking of neighbouring starch molecules through two hydroxyl groups, whereas in some cases, blending of synthetic polyester with a starch polymer is used to reduce its hydrophilicity. Thus, the removal of hydroxyl group is responsible for the formation of strong bonding between starch molecules and gives stability to the plastic. Hence, the formation of both biodegradable and non-biodegradable plastics from renewable resources is important to reduce the effect of petrochemical-based plastic and to save earth resources (Johansson et al. 2012).

12.4 Types of Biopolymers and Their Uses

12.4.1 Edible and Non-hazardous

The edible polymer which is used for coating of food material to extend longevity of food and food quality and safety is known as edible film coating. It prevents the food from the loss of moisture and makes it selectively permeable for exchange of gases. It also prevents food from the loss of its important components. The most common example of edible food coating is wax which is used on fruits to prevent loss of moisture and provide shiny surface to it. The properties and efficiency of edible films are dependent upon the film-forming material such as proteins, carbohydrates and lipids termed as natural biopolymer (Pavlath and Orts 2009).

12.4.1.1 Natural Polymer

Natural polymers are those substances which are obtained naturally. These natural polymers are produced from various natural substances such as proteins, polysaccharides and lipids. These natural polymers are formed either by the process of condensation of polymerization or addition of polymerization. The natural polymers are the most suitable component to make environmentally friendly bioplastics because of their functional properties like water permeability, gas barrier properties and mechanical properties like flexibility and elasticity. However, on application purpose, there is a need for improvement in mechanical strength and moisture susceptibility of these natural polymers (Khan et al. 2017).

The natural polymers which are broadly used in pharmaceutical and food packaging industry due to their biodegradability and sustainability may be classified as protein-based and polysaccharide-based polymer.

Protein-Based

Proteins are an excellent component of coating and film material. The protein contains charged polar and nonpolar amino acid group in its structure along with protein chain, due to which it has great chemical potential. The diverse amino acid functional group of protein contains multiple sites for chemical interaction. The stability of film coating can be improved by chemical alteration in protein structure. The cross-linking property of protein like binding of two or more protein molecules provides more stability. The hydrophobic property of protein-based films is considered commercially preferable in contrast to other biopolymer-based films. These protein-based films are completely decomposable. During degradation process, they release nitrogen, which acts as a natural fertilizer. Another important fact is that upon digestion, bioactive peptides are produced which have great health benefits such as antihypertensive and radical scavenging (Preeti et al. 2019). The bioactive compounds have numerous health benefits and perform an important part in enzymatic hydrolysis, microbial fermentation and gastrointestinal digestion. These bioactive compounds are also widely used by food and medical industries due to their high nutritional values.

It is commonly used in protection of food from microbial invasion which increases the longevity and quality of the product. It provides exceptional gas barrier property which helps to protect the high-fat food from being rancid and off-flavour due to high oxidation. Protein-based films are best known for coating of fresh fruits and eggs. It also protects the fruits from browning and ripening which plays an important role in increasing the shelf life of a product. When used as coating on food, it reduces the moisture loss and aroma loss of food material (Preeti et al. 2019).

Composition

Proteins obtained from living sources are used for the formation of bioplastic. It is comprised of three main components: proteins, plasticizers and solvents. The characteristics of protein films can be affected by the intrinsic factors like amino acid composition, crystallinity, hydrophobicity, hydrophilicity, molecular size, surface charge and three-dimensional shape of coating components or extrinsic factors (heat, dehydration, pH, moisture resistances during processing and pressure).The hydrophobicity of protein is due to the presence of high concentration of nonpolar amino acids (Preeti et al. 2019).

Types of Protein-Based Polymers

There are different types of proteins which can act as biopolymer, out of which the most used protein-based polymers are *whey protein*, *collagen*, *gelatine* and *soy protein*.

Whey Protein

The residue left in milk serum after the coagulation process of casein of pH 4.6 at 20 °C is termed as whey protein. It is basically a by-product of cheese production. Its basic structure is composed of bovine serum albumin, β-lactoglobulin, α-lactalbumin and immunoglobulins, which are all globular proteins. The whey protein denatures at high temperature or in alkaline medium. It carries negatively charged amino acids which are evenly distributed throughout the protein chain, and the folds of protein chain are embedded with hydrophobic group. The interaction between protein chains determines the properties and formation of film network (Schmid et al. 2012; Preeti et al. 2019; Dangaran et al. 2009).

Uses of Whey

It consists of important amino acids for human body as it possesses great absorbance property. It is widely used in the treatment of lactose-intolerant patients. It is mostly used in food supplements as it helps in muscle protein synthesis and promotes lean muscle mass growth. When used as coating on foods, it reduces the moisture loss and aroma loss and provides permeability of oxygen and carbon dioxide gas.

Collagen

Collagen is a structural fibrous protein found in all animals. It comprises of three parallel α-chains which combine to form super-helical structure. Collagen contains amino acid in its structure, which are basically repeating trio of glycine residue with proline and hydroxyproline. The collagen polymer has great thermal stability, elasticity and good mechanical strength which make it feasible in the production of biofilm (Haug et al. 2004).

Uses of Collagen

Collagen is used in supplements as it improves the skin appearance and elasticity by minimizing lines and wrinkles. It is also used in medicinal supplements to cure posttraumatic osteoarthritis (PTOA). It also promotes the muscle mass in human body. It is used in shampoo for hair growth and thickness. The collagen provides strength and flexibility to the plastic. When used in food, coating materials protect it from browning and ripening.

Gelatine

When mild heat treatment is given to collagen under acidic or alkaline condition, gelatine is formed. However, during the hydrolysis of collagen, it partly gets denatured, but upon chilling, it partially regains its structure. The resulting protein material is called gelatine. Gelatine contains a broad range of essential amino acids in its structure. Gelatine has good gelling properties, so it is used in the food industry as thickening and stabilizing agent. Gelatine has an excellent foaming property that helps to form bioplastic which can be used by pharmacological and nutraceutical industries (Preeti et al. 2019; Dangaran et al. 2009).

Uses of Gelatine

The use of gelatine in diet improves the brain functioning and improves the skin appearance and joint problems. It is a useful gelling agent in food production such as in jellies and gummy candies. It can be consumed as broth or as supplement. It is used for the specific application like in food coating. It protects the food from bacterial invasion. It also increases the longevity and maintained quality of edible goods like fresh fruits and eggs. It also protects the fruits from browning and ripening.

Soy Protein

Its basic structural unit is globular protein. The two main organizational units of soya proteins are β-conglycinin and glycinin which build about 37% of soya protein structure. The glycinin in soya protein shows excellent gelling and foaming properties. The film formation of soya protein is affected by high temperature or under alkaline condition as the soya protein becomes denatured. The denatured protein forms disulphide bonding which disturbs the tensile strength of film. The stability of Soya Protein films depends upon the ionic strength of molecules (Kunte et al. 1997; Cho and Rhee 2004).

Uses of Soy Protein

There are various dietary benefits of using soy protein as it prevents from cardiovascular diseases and obesity. The soy protein is good for the health of people who are suffering from high cholesterol problem as it helps to maintain low-density lipoproteins (LDLs) and triglycerides. It is good for vegetarian people who don’t eat meat and egg for various reasons, and they can use soy protein as its substitutes. It is also used in fresh food coating as it is found effective in delaying lipid oxidation. It is also used in fruit and vegetable coating. It increases the longevity of food.

Polysaccharide-Based Edible Films and Coatings

They are soluble in water and form strong hydrogen bonding. Because of the size, molecular mass and arrangement of their molecules, these polysaccharides have an ability to form gel in water, due to which polymer chains and intermolecular fragments strongly bind with each other. In nature, polysaccharides are the most abundantly found natural polymer such as cellulose, starch, alginates, agar, carrageenan and pectin. Starch is the most ubiquitous polymer whose films are often transparent, translucent, odourless, tasteless and colourless. It is used in wrapping food material due to its edibility. Starch improves the tensile strength, moisture absorption and oxygen permeability (Preeti et al. 2019).

Cellulose

It is the most abundant polysaccharide found in cell wall of plant. It helps in maintaining the shape of plant cell and provides strength to cell structure. Cellulose is indigestible by all animals including humans but can be digested by microorganisms that are present in the intestinal tract of animals. Commercially the cellulose is mainly obtained from wood which is comprised of 50% of cellulose. It is also obtained from cotton which provides 90% of cellulose in dried form. Cellulose is also produced commercially by the fermentation of bacteria and through chemical modification of cellulose molecule into cellulose acetate, which is used in different products. However, the chemical alteration or modification in cellulose structure reduces the biodegradability of polymer in soil and water to some extent. The cellulose polymer has a strong hydrogen bonding that makes it a stronger fibre as compared to starch polymer which is widely used in biofilm production for various applications (Ashok et al. 2016; Hazrati et al. 2019; Hossain et al. 2016; Ul-Islam et al. 2016; Agustin et al. 2014).

Uses of Cellulose

Cellulose is used as the edible coating in ready-made food products which contain modified cellulose polymer (Preeti et al. 2019). Cellulose is commercially used on a large scale for paper formation. The cellulose which is chemically modified can be used in the formation of paints, plasters, adhesive, ceramics, cosmetics, pharmaceutical film coating and numerous other products.

Starch

Starch is an abundant hydrocolloid carbohydrate component on earth. Starch functions as energy storage in plant cell. When it is metabolized by plant cell, the energy is released which is used for plant growth. The basic structure of starch consists of organic compounds, i.e. CHO in the ratio of 6:10:5. The amylose and amylopectin are the two major polymer components of starch, and their amount varies according to plant type. The amylose content of starch maintains elongation strength of starch films. In order to increase the yield of amylose and amylopectin, we can use genetic modification, e.g. in corn starch, 28% amylose and 72% amylopectin are present which after genetic modification increase up to 85% of amylose and 100% of amylopectin which can be used for all practical purposes (Ashok et al. 2016; Jiménez et al. 2019). Its polymer has low mechanical property which leads to poor film-forming capacity, but it can be overcome by plasticization. Water is considered as a primary plasticizer for starch as the molecular bonding of starch is changed upon heating with water. It affects the oxygen permeability of starch film and at low humidity it shows excellent oxygen barrier property. Due to these properties, the starch films are widely used in biofilm formation (Ashok et al. 2016; Jiménez et al. 2019; Khalil et al. 2017; Shaikh et al. 2019; Mehboob et al.

2020). The annual production of starch is 70 billion pounds or more. The starch is mainly produced from sweet potato, corn, wheat and other resources. The overall half of the starch produced is partially hydrolysed to produce various edible products.

Uses of Starch

The partially hydrolysed starch is used in manufacturing of sugar, corn syrup and products like wine or as feedstock for different industrial processes such as biochemical, pharmacological and fermenting industries. It is also used in food industries in the manufacturing of bread butter, baby food, cakes, confectionaries, pudding, glaze and sauces.

Alginate

Alginate is a hydrocolloidal substance that is extracted from brown seaweeds such as *Sargassum* spp., *Iyengaria* spp. and *Colpomenia* spp. for the formation of edible films. It is a lined binary copolymer that consists of two 1,4-linked polyuronic acids (Lim et al. 2018). The polyuronic acid consists of three types of building blocks, which are G-block (α-L-guluronic acid), M-block (β-D-mannuronic acid) and both M&G blocks (Draget and Taylor 2011). The extraction process of alginate is usually done by using Na_2CO_3 (Pegg 2012). The alginate forms the hydrophobic gel in the presence of multivalent metallic cations, e.g. calcium ion. The hydrophobicity is an important characteristic which shows strong molecular bonding between carboxyl groups of guluronic acid with Ca^{2+} that forms 3D gel-like structure (Sellimi et al. 2015). The alginate films show excellent water-resistant property in combination with plasticizer (Niaz et al. 2020; Ahmed and Butt 2014; Younas et al. 2014).

Uses of Alginate

Alginate-based films are good oxygen barriers. The alginate films are resistant to plant diseases; hence, they are nutritionally significant. It is also used as thickener in food industries such as thickener in salad dressings. It is also a very good antimicrobial agent.

Carrageenan

Carrageenan is one among the foremost promising phycocolloids extracted from red seaweeds, which shows brilliant film-forming ability (Paula et al. 2015; Siah et al. 2015). The basic structure of carrageenan is a linear chain of carbohydrate shaped by alternate units of D-galactose and 3,6-anhydrogalactose joined by α-1,3- and β-1,4-glycosidic linkages. It is conjointly contained 15–40% ester sulphate bond with a median mass higher than 100 kDa. It also contains methoxy and pyruvate groups. The polymer films of carrageenan show poor water-resistant property and water vapour permeability (WVP); as a result of that, they are termed as hydrophilic in nature. The hydrophilicity of carrageenan polymer restricted its application in food and pharmaceutical industry (Shojaee-Aliabadi et al. 2014; Alves et al. 2006).

Uses of Carrageenan

It is used in a variety of foods as it provides antimicrobial and antioxidant property. It also reduces the oxidation rate, loss of moisture and breakdown of food. It is used in confectioneries, puddings and creams and cakes. It is also used in coating of medicines and drugs by pharmaceutical industries.

Agar

Agar is derived from a variety of red seaweeds like *Gracilaria* sp., *Gelidium* sp., *Champia* sp., *Asparagopsis* sp., *Coelarthrum* sp. and *Melanothamnus* sp. It is a polysaccharide found in the inner part of red algal cell walls belonging to the family Gelidiaceae, Gracilariaceae, Gelidiellaceae and Pterocladiaceae (Lee et al. 2017a, b; Marinho-Soriano and Bourret 2005). Agar is composed of 1,3-linked D-galactose and 1,4-linked 3,6-anhydrous-L-galactose units and side chain changed by sulphate ester, methoxy group and pyruvic acid residues. The gel quality and amount of agar vary as per species due to external growth factors such as change in temperature, salinity of water and nutrient content (Lee et al. 2017a, b). Agar has higher elasticity as compared to hemicellulose due to ecological strong ocean wave action (Ficko-Blean et al. 2015). Agar has diverse applications in food material, medicines, cosmetics and therapeutic and biotechnology industries. The agar is a carbon-containing eco-friendly polymer and has now been used in the formation of biofilms (Gade et al. 2013; Machmud et al. 2013; Wu et al. 2009; Leceta et al. 2014; Tabassum 2016; Hira et al. 2018). Moreover, plasticizers are also added to decrease the breakability of polymer, and they work like a fragment between polymer chains (Mekonnen et al. 2013). It increases the flexibility and extensibility by reducing intermolecular forces between polymers (Abdorreza et al. 2011; Fakhouri et al. 2012; Wu et al. 2009; Leceta et al. 2014).

Uses of Agar

Agar is used in confectionaries, dessert, beverages and ice creams. It is also used in different kinds of seafood like in sushi and used in pet food. It is also used in coating of drugs and medicines. Now it is also rarely used in disposable crockery. It is extensively used in laboratories as culture medium.

Pectin

The heteropolysaccharide pectin (PEC) is the basic element of cytomembrane of all plants and few seaweeds. It is considered as a complex biomacromolecule because it maintained the integrity and stiffness of plant tissues by inducing both cell-cell adhesion and mechanical strength (Jolie et al. 2010). The characteristic structure of pectin is a linear chain of α-1,4-linked d-galacturonic acid residues. It is esterified with methoxylated residues which are often present in either low (LMPEC) or high methoxylated PEC (HMPEC) percentage (Sila et al. 2009). However, the chemical composition of PEC differs from plant species to species. Thus, PEC heterocompound organization is still a theme of discussion, and its precise organic compound has not been all explained clearly (Esposito et al. 2016; Coenen et al. 2007; Vincken et al. 2003).

Uses of Pectin

Pectin is known for being an excellent film-forming polymer. Pectin is used in making biodegradable straws. The use of pectin and starch can result in the formation of strong self-supporting films. The pectin polymer-based films are strong, flexible and thermally stable up to 180 °C.

12.4.2 Non-edible and Non-hazardous

The biodgradable and compostable polymers cannot be used for edible purposes as they are toxic to human body. It can be utilized in various fields such as in surgical aids and medicinal product. The material of these plastics is eco-friendly, therefore providing a wide range of usage without harming the environment.

12.4.2.1 Biochemosynthetic Polymer

Many monomers which are obtained from living resources can be polymerized, and the resultant polymer may be known as biochemosynthetic polymer. The polymers are not produced by living body itself, but they can be synthesized by polymerization of monomers separately from living organism. They are regarded as biodegradable biopolymers. The amino acids, lactic acid and various triacylglycerols are some examples of biochemosynthetic polymers.

Polylactic Acid (PLA)

PLA is a polyester which has similar mechanical properties with polyethylene polypropylene and polystyrene. The basic building block for PLA is lactic acid (2-hydroxypropionic acid), which is known as naturally occurring three-carbon chiral acid (Johansson et al. 2012). The PLA is a renewable bioplastic derived from starch and sugar. These plastics are breakable below a various environmental condition except PLA hydrolytic condition which required relatively high temperature such as 60 °C. However, the degradation rate of this plastic is controlled by alteration in its monomer arrangement and crystallinity (Sudesh and Iwata 2008). It is an adaptable polymer, is recyclable and degradable, has high molecular mass and smart processability and is water-resistant. PLA is a copolymer between poly-L-lactic acid and poly-D-lactic acid. Counting on position of L-lactide/D-lactide, PLA properties vary from semi-crystalline to amorphous. Mostly the amorphous form of PLA which contains about 12% of D-lactide is treated by thermal moulding, which is a technology used in the packaging industries, and it shows properties like styrene. An international company is formulating PLA based plastic product with short shelf life (Bugnicourt et al. 2014; Siracusa et al. 2008). PLA and its copolymer have great application in pharmaceutical and biomedical field. It can be used in the form of biomedical junctions and mediums for drug transfer. The polylactic acid plastic is an emerging thermoplastic material that has acquired much consideration as the biopolymer (Hazrati et al. 2019; Ashok et al. 2016; Sudesh and Iwata 2008).

Use of PLA

PLA is now being manufactured worldwide. PLA is now being manufactured by many companies worldwide and are considered as leading business tycoons. The PLA films have been processed into packaging material like platters for wrapping of salads, fast food and delicatessen food. It is also used for medical and textile purposes. These products are now available in North American and European countries. PLA has been also used in manufacturing of non-carbonated beverage bottles, e.g. water bottles (Ashok et al. 2016; Johansson et al. 2012). PLA is also used in the manufacturing of mobile phones, notebook computers as well as headset and sound system. Some automobile companies used PLA in the manufacturing of car parts such as tyre, covers, etc. (Sudesh and Iwata 2008). PLA is also used in the coating of disposable cups of hot drinks using additives (plasticizers or impact modifier). Instead of this commercial advancement in PLA products, there is still a necessity of economic methods to improve PLA properties in terms of water resistance, reduced fragility and increased thermal stability (Johansson et al. 2012).

12.4.2.2 Biosynthetic Polymer

The biopolymers which are made by using microorganisms mainly bacteria through the process of fermentation are called biosynthetic polymer. The microorganisms convert a supplied substrate into polymers, which are then extracted and purified. The production of biosynthetic polymer on a large commercial scale has been recently developed.

Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates are an important polymer that has great potential as an alternative substitute for petrochemical-based plastic due to its biodegradability and rubber-like properties. It also has a better oxygen barrier and water barrier property as compared to non-biodegradable polypropylene (PP). The PHA and its copolymer due to their physicochemical properties are used in various fields including food packaging and medical applications. The raw materials used for PHA formation are food crop, sugarcane, rice, cassava, wheat, sweet potato, potato, corn and vegetable oil (Ashok et al. 2016; Rafique et al. 2019; Tsang et al. 2019).

The process of PHA includes the following steps: substrate preparation, PHA-accumulating fermentation and PHA extraction. The physicochemical properties are determined according to the operating parameter and the bacterial species used for the fermentation process. The biopolyester, the PHA produced by controlling the growth of bacteria under nutrient-limiting condition, is mainly genus *Lactobacillus* used in the fermentation process as it can produce increased amount of lactic acid from sugar. There are around 250 types of PHA producers which have been discovered, out of which few bacteria species have been adopted to produce PHA (Rafique et al. 2019; Sudesh and Iwata 2008; Bugnicourt et al. 2014). The PHA is widely used for packaging material, medical application, energy and fine chemicals.

Use of PHA

The PHA are biocompatible and decomposable and have medical application in the area of surgical sutures, controlled drug release and others. These natural polyester polymers are substantial feedstock for bioplastic production. According to recent research, there are some corporations which are practising PHA for profit orientation. These corporations can yield PHA of about 10,000 tonnes/annum. Many of these corporations are struggling to become a staple supplier of PHA.

12.5 Conclusion

In this era, the world is moving towards the sustainable packaging which can provide reduction in the waste, less consumption of raw material used for plastic making and reduction in transportation cost. The scientists are using biodegradable or renewable polymer with synthetic or non-synthetic material to increase the stability of material. The biopolymer market is becoming competitive day by day as it is used in edible coating and film forming in various food materials due to its numerous advantages like reduction in moisture loss and excellent gas barrier properties. However, in current situation, the biopolymer is in its progressive stage, but it holds a promising future in developing a sustainable bioplastic industry. Although the price of bioplastic is twice as compared to synthetic plastic, the advancement and progress in the production of bioplastic might have potential to improve environmental sustainability and in reshaping the economic condition as compared to synthetic one. The scientists from different fields work on production of bioplastics from different kinds of biopolymers like natural biopolymers, synthetic biopolymers and chemosynthetic polymers. The countries which have already developed the bioplastic industries includes the United States as well as European countries like France, Germany, England, the Netherlands, Italy, Austria and Spain and Japan and China (Johansson et al. 2012; Siracusa et al. 2008). Many papers have also been published from Asia on the bioplastic and its use in packaging industry (Rajendran et al. 2012; Khalil et al. 2017). Similarly, many research papers have been stated from Pakistan on the utilization of biopolymer in the production of bioplastic (Rafique et al. 2019; Khan et al. 2017; Mehboob et al. 2020; Shaikh et al. 2019; Tabassum 2016; Hira et al. 2018). However, there is still a need for research in the field of bioplastics as they show restrictions in performances like heat resistance, water permeability, gaseous exchange, flexibility, etc. which helps to improve the quality, longevity and microbiological safety of biopolymer-based food packaging.

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Biocomposites of Polyhydroxyalkanoates and Lignocellulosic Components: A Focus on Biodegradation and 3D Printing

13

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Abstract

Polyhydroxyalkanoates (PHAs) are defined as natural and biodegradable biopolymers obtained by microbial synthesis. PHAs are classified as short chain length (3–5 carbon monomers), medium chain length (6–14 carbon monomers), and long chain length (15–18 carbon monomers), depending on the number of carbons in the monomeric constituents. Little is known about the biodegradation potential of PHAs in nature and how this can be tailored by adding complementary polymers and lignocellulosic components. In this chapter, we have reviewed the current literature with a focus on biocomposites based on PHAs and lignocellulosic materials. We also complemented this chapter with a case study, including recent results from our research activities within this area. Examples are provided on how lignocellulosic materials affected the mechanical properties of PHAs and increased the biodegradation rate in soil. We also explored the potential of 3D printing by fused deposition modeling as a novel technology to manufacture PHA-based products.

Keywords

Biodegradable bioplastics · 3D printing · Medical orthosis · Thermomechanical pulp · Polyhydroxyalkanoates/fiber composites · Biocomposite inks

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13.1 Overview of Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are aliphatic biobased and biodegradable polyesters obtained by microbial synthesis. The PHA family is formed by numerous polyesters differing in chain length, short chain length (3–5 carbons), medium chain length (6–14 carbons), and long chain length (15–18 carbons) (Volova et al. 2020), which exist either as homo- or copolymers (Ross et al. 2017). In total, some 150 PHA monomers have been identified. The short-chain-length PHAs are highly crystalline thermoplastics, while the medium-chain-length PHAs are elastomeric. The short-chain-length PHAs include polyhydroxybutyrate (PHB), which is the most common polyester of the PHA family, and poly(3-hydroxyvalerate) (PHV), or their copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). The long-chain-length and medium-chain-length PHAs are uncommon and less studied. The medium-chain-length PHAs include poly(3-hydroxyoctanoate) (PHO) and poly (3-hydroxynonanoate) (PHN) (Li et al. 2016; Raza et al. 2018), while the long-chain-length PHAs are poly(3-hydroxypentadecanoate) (Tan et al. 2014).

The PHAs are synthesized by bacteria, archaea, and microalgae and accumulate as an intracellular reserve material to be used as a source of carbon and energy. The reserve of PHA is in granule form in the microbial cytoplasm and once extracted and purified present thermoplastic properties. PHB was discovered in 1926 as a constituent of the bacterium *Bacillus megaterium* (Poirier et al. 1997). The *Ralstonia eutropha* is the most common bacteria to produce PHB, which can store up to 90% in dry weight of medium-chain-length hydroxyalkanoic acids obtained from an axenic culture (Steinbüchel and Lütke-Eversloh 2003). Other bacteria that synthesize PHBs are *Bacillus mycoides*, *Bacillus sphaericus*, and *Cupriavidus necator* (and variants). The substrates applied as a carbon source for PHB production are glucose, saccharose, starch, glycerol, and plant oils (Alves et al. 2017). The copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) using *Ralstonia eutropha*, *Cupriavidus necator*, and *Alcaligenes eutrophus* bacteria uses propionic acid (Kobayashi et al. 2000; Marangoni et al. 2002), fatty acids, levulinic acid (Kachrimanidou et al. 2014), pentanol (using *Paracoccus denitrificans* bacteria) (Yamane et al. 1996), citric acid, or amino acids as substrate (Steinbüchel and Lütke-Eversloh 2003).

The bioplastic production in 2019 was around 2.1 million tons, and PHA production was about 1.2% of the total production. The estimated production capacity of biodegradable bioplastics (polylactic acid (PLA), PHA, and starch) for the next 5 years is 1.3 million tons, and PHA production is expected to triplicate its volume. The packaging (flexible and rigid) market is the main consumer of bioplastics (53% of the total produced), but PHA has only been used for flexible packaging applications within this sector (European Bioplastics 2019).

13.2 Lignocellulosic Waste as a Substrate to Produce Polyhydroxyalkanoates

The carbon source significantly affects the economics of PHA production (Choi and Lee 1999). For this reason, alternatives are currently being sought that include highly available raw materials from renewable and low-cost resources. The lignocellulosic materials are the most promising resources to be applied in the PHA production. The alternatives involve biomass wastes from production processes or crops. The wood industry generates sawdust, shavings, and bark, usually burned and used for heat and electricity generation or stored in the same place where it is generated (Area and Vallejos 2012; Vallejos et al. 2016). On the other hand, bagasse, husks, and straws are wastes from processes or crops. Salgaonkar and Bragança (2017) studied the production of PHAs from sugarcane bagasse (SCB) using *Halogeometricum borinquense*. The PHA productions were 50.4% for 20% SCB/80% nutrients and 45.7% for 50% SCB/50% nutrients. Thermogravimetric analysis (TGA) showed the existence of multiple melting peaks in the polymers and indicates a variation in monomer units (Salgaonkar and Bragança 2017). Additionally, proton nuclear magnetic resonance (H-NMR) analysis demonstrated the presence of methyl groups from 3HB (peak at 0.889 ppm) and 3 HV (peak at 1.26 ppm). The pretreated SCB allows a better action on sugars (Getachew and Woldesenbet 2016). The use of pretreated SCB as a substrate for *Bacillus* sp. reached a production of 55.5% of P3HB, while the use of the hydrolysate of the SCB and *Ralstonia eutropha* produces 56.7% (50% hydrolysate SCB/50% nutrients), 60.2% (75% hydrolysate SCB/25% nutrients), and 56.5% (100% hydrolysate SCB) of PHA.

Pinto-Ibieta et al. (2020) studied the effect of using synthetic hemicellulose hydrolysates (SHH) rich in xylose as a carbon source to feed mixed microbial cultures. The SHH simulated the composition of liquor from the hydrothermal treatment of sugarcane bagasse. The simultaneous biological production of levulinic acid (LA) and PHAs were possible, reaching a maximum of 14% and 17% w/w, respectively, using a substrate rich in acetate. Thirty-two percent of LA and 3% of PHB were obtained when using a substrate rich in xylose (Pinto-Ibieta et al. 2020). The lower PHA yields obtained may be attributed to inhibitors or limitations of the biologically available xylose (present as xylan oligomers) (Keenan et al. 2006). Diluted acid and enzymatic hydrolysis were used to prepare a hydrolysate of spruce sawdust (SSH) substrate (Kucera et al. 2017). The SSH was treated with *Burkholderia cepacia* and *Burkholderia sacchari*. Activated charcoal and overliming were used to separate the inhibitors. The PHB content reached values up to 90% with previous detoxification of the SSH, whereas only 10% content without SSH detoxification. Besides, the authors applied lignite powder as a reagent to eliminate the inhibitors with similar results as activated charcoal. In the same way, Silva et al. (2007) studied the production of PHBV from pretreated *Pinus radiata* sawdust using H_2SO_4. The hydrolysate was treated with *Brevundimonas vesicularis* and *Sphingopyxis macrogoltabida*, and the PHA content was 64% and 72%, respectively. Detoxified aspen wood-based hemicellulosic hydrolysate and maple-based hemicellulosic hydrolysate were used as a substrate to produce PHBV by

Burkholderia cepacia (Keenan et al. 2006). In the culture medium, 0.45 w/w% and 0.6 w/w% of levulinic acid were added as a co-substrate in the detoxified aspen-based and the maple-based hemicellulosic hydrolysate, respectively. The produced PHBV was 40% and 39% for the aspen hydrolysate and the maple hydrolysate, respectively. Other raw materials studied as carbon sources to produce PHAs are rice husk, banana peel, grass biomass, corn stover, coir pith, oil palm, and spent coffee (Al-Battashi et al. 2019). In all cases, the production requires pretreatment of the biomass (H_2SO_4, NaOH, or enzymes). PHB and PHBV were produced using a rice husk hydrolysate in combination with several organic nitrogen sources (gluten hydrolysate/soybean meal/soy peptone/oatmeal) and *Bacillus mycoides*. The rice husk hydrolysate, in combination with a gluten hydrolysate, resulted in a maximum synthesis of 50% of PHB and 34.5% of PHBV in presence of valeric acid (Narayanan et al. 2014).

13.3 Wood/PHA Biocomposites and 3D Printing: Defining a Case Study

3D printing or additive manufacturing facilitates the fabrication of personalized parts that usually demand higher costs and longer periods when manufactured by conventional methods. In recent years, numerous studies have been carried out on the use of fossil and biobased plastics for 3D printing. In this section, we describe a case study to provide examples of the properties and characteristics of wood/PHA biocomposites to be applied in 3D printing (Fig. 13.1). Figure 13.1 shows the methodology applied to obtain the 3D structures, from the production of the filament by extrusion to the printing of the 3D structure and the subsequent biodegradation analysis. Filaments for 3D printing were obtained from mixtures of polyhydroxyalkanoates (PHAs) and lignocellulosic fibers produced from pine, and 3D printed structures were manufactured as examples. These filaments were characterized to determine water absorption and mechanical and thermal behavior. Finally, 3D structures were manufactured using fused deposition modeling (FDM), and biodegradability studies were carried out to evaluate the degradation capacity in soil. Thermomechanical pulp (TMP) fibers provided by Norske Skog Saugbrugs were used as reinforcement. The polymers were purchased from GoodFellow Cambridge Limited, in granules with a nominal size of 3 mm. The polymer matrix was polyhydroxyalkanoate (PHA), and the melt flow rate (MFR) was 3. The samples were compared with a commercial PHA biocomposite containing wood flour (MFR = 4), purchased from GoodFellow. The samples assessed in this study are shown in Table 13.1. Water absorption and mechanical and thermal properties of filaments ($d = 1.75$ mm) were assessed. The filaments were used to print 3D structures for further biodegradation assessment. The method to produce a 3D structure by FDM from fibers and polymers include a previous blending of components and a two-step extrusion (Filgueira et al. 2017).

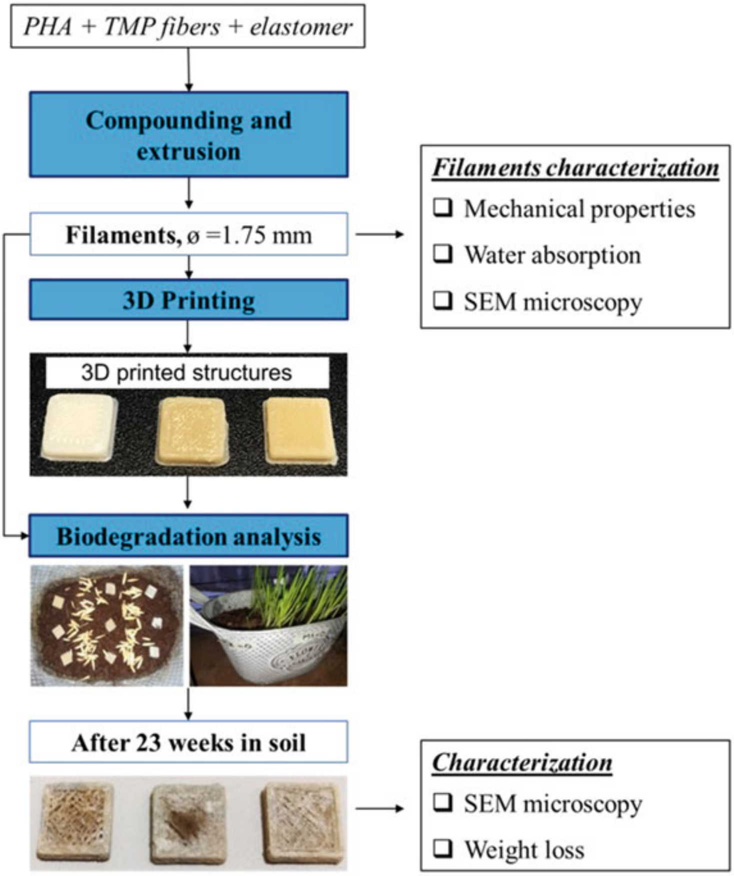

Fig. 13.1 Overview of the production and characterization of the 3D structures

Table 13.1 Blends used for manufacturing filaments in this study

Code	PHA (wt%)	TMP fibers (wt%)	Elastomer (wt%)
PHA	100	–	–
20TMP	80	20	–
20TMP5E	75	20	5
Commercial	u.n.	u.n.	u.n.

u.n. uninformed values

13.3.1 Mechanical Properties of Filaments

The tensile properties of biocomposites with PHAs and fibers were previously studied (Singh and Mohanty 2007; Singh et al. 2008; Sánchez-Safont et al. 2018). The addition of fibers to PHB or PHBV matrices is expected to increase the tensile properties. The tensile strength depends on the compatibility between the matrix and

Table 13.2 Tensile strength (σ), Young's modulus (E), and elongation at maximum strength ($\varepsilon_{\text{máx}}$) of filaments

	σM^2 (MPa)	εB (%)	E (MPa)
PHA	14.6 ± 2.2	19.1 ± 2.3	501 ± 44.0
20TMP	16.5 ± 2.9	18.6 ± 2.4	605 ± 63.4
20TMP5E	17.3 ± 1.6	19.6 ± 1.9	505 ± 37.9
Commercial	15.6 ± 2.3	19.1 ± 1.1	704 ± 75.8

the fibers, whereas the *E* modulus depends on the fiber impregnation and aspect ratio (Bhardwaj et al. 2006). Previously we have demonstrated that the addition of TMP fibers enhances the mechanical properties of PLA (Filgueira et al. 2017) and biobased polyethylene (BioPE) (Tarrés et al. 2018). TMP fibers also improved the printability of BioPE (Filgueira et al. 2018). The mechanical performance of the filaments manufactured in this case study is given in Table 13.2, which shows that the addition of TMP fibers as reinforcement in PHA matrices increases the tensile strength with 13.0% (20TMP) and 18.5% (20TMP5E) with respect to 100% PHA. The lowest value in tensile strength was measured for the commercial filament, having only a 6.8% increase. The obtained values are similar to biocomposites of PHBV in combination with 40% of wood flour (Singh and Mohanty 2007) or bamboo fiber (Singh et al. 2008). However, the use of 20% of bamboo pulp (Jiang et al. 2010) or 40% of recycled cellulose fiber (Bhardwaj et al. 2006) reached higher tensile strength values. However, Singh et al. (Singh et al. 2008) reported a decrease in the tensile strength of PHBV biocomposites with an increase of wood flour. The tensile strength decreased by 20%, with an addition of 40% wood flour due to the lack of sufficient interfacial interaction between the fiber and matrix (Singh and Mohanty 2007).

The values for *E* modulus were higher for the biocomposites than the sample with 100% PHA. The commercial sample reached the highest increase. However, the values were lower than those obtained using bamboo fibers and pulp (Singh et al. 2008; Jiang et al. 2010) or wood fibers (Singh and Mohanty 2007). In comparison with other matrices, the tensile strength and *E* modulus values for samples with 20% TMP fibers were lower than those using BioPE (Filgueira et al. 2018; Ehman et al. 2020), PP (Stark and Berger 1997), and PLA (Filgueira et al. 2017; Sood and Dwivedi 2018) as the matrix. An increase of the *E* modulus (up to 2.9 GPa) in samples of PHA reinforced with 20% fibers (pine sawdust) was obtained using a plasticizer (acetyl-butyl citrate) and calcium carbonate (Cinelli et al. 2019). Ductility is an interesting property that, in most cases, is reduced when adding natural fibers to bioplastics due to the brittle characteristic of the resulting biocomposites. Compared to the sample without elastomer (20TMP) that was more brittle, the tensile strength and elongation increased when adding an elastomer (20TMP5E, Table 13.2).

Scanning electron microscopy (SEM) is a valuable and versatile characterization tool commonly applied in polymer science. SEM provides a three-dimensional image with high resolution and is used to characterize the morphology, surface, particle size, mechanical ruptures, defects such as cracks, etching residues, differential swelling, depressions, and perforations (Rydz et al. 2019). SEM images showing

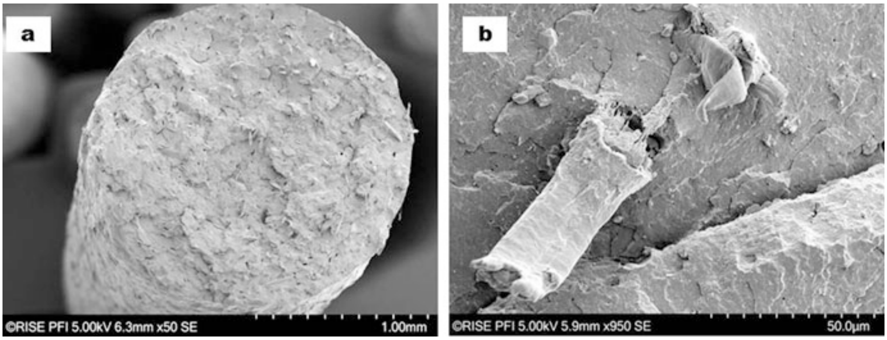

Fig. 13.2 SEM images of the rupture point from tensile tests of filaments. Image magnification: (**a**) ×50SE and (**b**) ×950SE

cross-sectional views of the filaments at the fracture area, after the mechanical testing, are shown in Fig. 13.2a. A heterogeneous structure with fibers distributed along the width of the filament was observed. The fibers were mostly at 0°, with respect to the direction of the force applied during tensile testing. A rough surface with a large number of pores was also observed. A closer view of the surface revealed the distribution and rupture points of some fibers (Fig. 13.2b). The presence of pores in the filament is lower than those reported using TMP fibers and BioPE (Filgueira et al. 2018). The high porosity in filaments of TMP/BioPE is associated with a lack of adhesion of superficial OH groups of cellulose and the hydrophobicity of the matrix. The result of the poor adhesion causes a reduction of mechanical properties and can be avoided by modifying the surface groups of the fibers or using compatibilizers (Cinelli et al. 2019).

A typical compatibilizer is a non-polar polymer grafted with anhydride groups. The reactive polymers obtained are frequently used for the compatibilization of non-polar polymers with polymers containing a large number of hydroxyl groups. The mechanism involves radical reactions using peroxide initiators (Imre and Pukánszky 2013). Usually, compatibilizers have been developed for increasing the interfacial adhesion between polyolefin polymers and natural fibers (Husseinsyah et al. 2013; Filgueira et al. 2018). However, such compatibilizers are based on fossil-based components such as polyolefin and maleic anhydride (MAH). We have recently demonstrated that MAH-grafted compatibilizers can also be biobased (Ehman et al. 2020). The grafting reaction of PHBV (Fig. 13.3) occurs in two stages: the production of the alkyl radical by peroxide initiation and the grafting of maleic acid (MA) to PHBV chains (Montanheiro et al. 2016). The compatibilizer is composed of two parts, i.e., the PHBV chain and the functional group that is then combined with the cellulose hydroxyls. Montanheiro et al. (2016) produced compatibilizer from PHBV and MA in a torque rheometer at 175 °C. The compatibilizer showed the same thermal degradation behavior, but a difference in temperature of crystallization (20 °C difference).

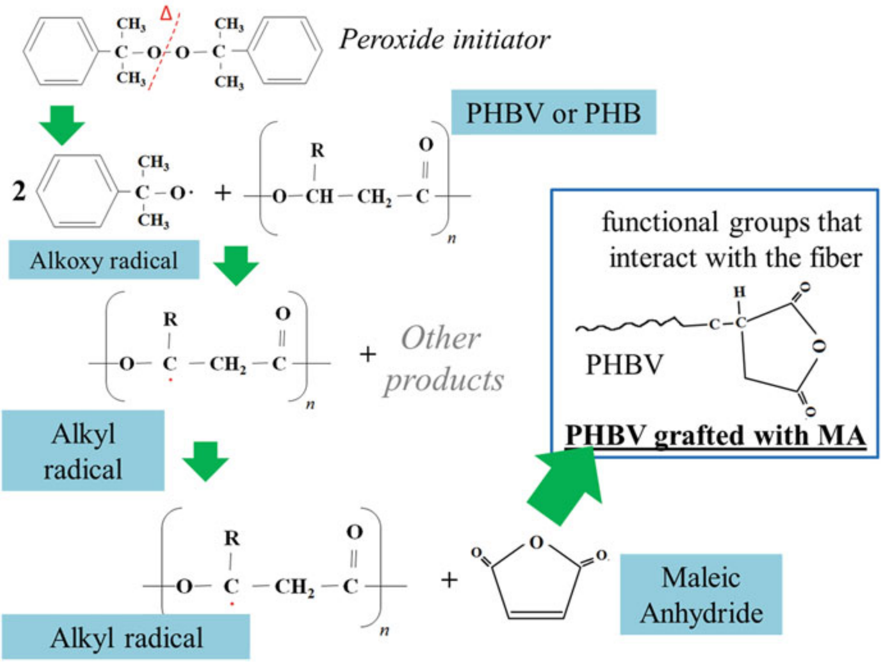

Fig. 13.3 Chemical reactions involved in the production of PHBV grafted with MA

13.3.2 Water Absorption of Biocomposites

The influence of water absorption on mechanical behavior when the cellulosic fiber is added to the matrix has been studied (Yang et al. 2006; Krishnaiah et al. 2018). The inclusion of cellulosic fibers increases water absorption (Chan et al. 2019), which is an important parameter to evaluate in biocomposites. Water absorption increased with the fiber content in matrices of high-density polyethylene (HDPE) (Ehman et al. 2020) and polypropylene (PP) (Espert et al. 2004). The presence of fibers produces micro-voids generating poor interfacial bonding and decreasing tensile strength (Yang et al. 2006). On the other side, water causes localized stress zones that provide pathways to access for bacteria and fungi, which thus promotes biodegradation (Chan et al. 2019). Three filaments of each sample (Table 13.1) were dried for 4 h at 50 °C, and the initial weight was determined. Samples were put in containers and immersed in distilled water at 25 °C. Water absorption was measured after 24 h, 96 h, 144 h, and 168 h. Results show that water absorption in all samples increased with time (Fig. 13.4). The 100% PHA sample showed two phases: an initial fast absorption stage (first 24 h) and a slower absorption thereafter. Similar behavior was quantified for the 20% fiber-80% PHA filaments. Important increments were shown at 96 h by the samples containing TMP fibers, elastomer, and PHA. Finally, the water absorption of the commercial sample continued to increase, even at the end of the trial (at 168 h). The obtained water absorption values are lower than

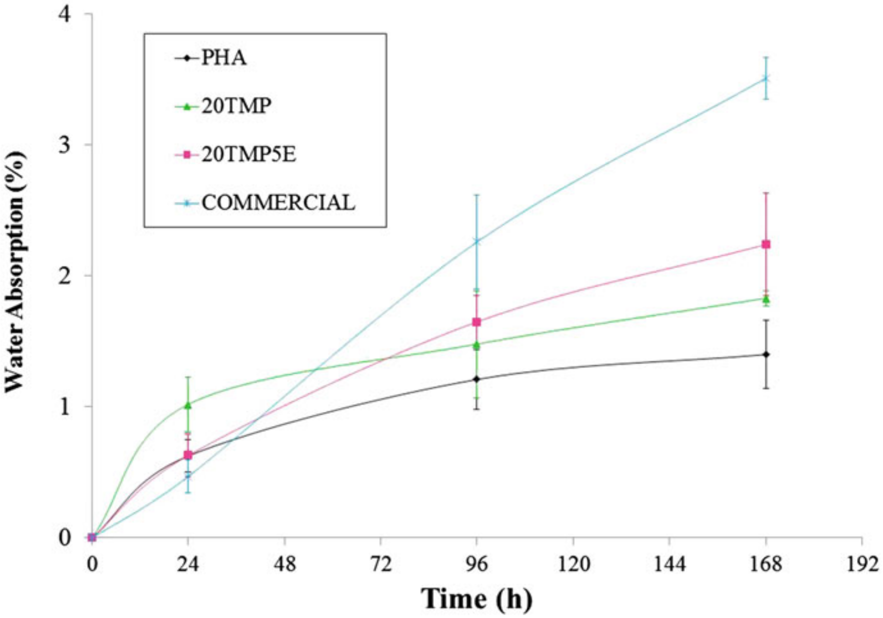

Fig. 13.4 Water absorption as a function of the immersion time in distilled water at 25 °C

those reported by Filgueira et al. (2017) using 20% of TMP fibers with enzymatic modification and PLA (5% water absorption after water immersion for 168 h). Also, filaments made by BioPE matrices reinforced with enzymatically modified 20% TMP fibers reached values of 12% of water absorption after 168 h immersion (Filgueira et al. 2018). The increase in fiber content increased the water absorption of the samples. Biocomposites of BioPE with 20% sugarcane bagasse fibers increased water retention by up to 6% (Ehman et al. 2020).

13.3.3 Thermal Stability and Decomposition Temperature of Biocomposites

Thermal analysis, such as TGA and differential scanning calorimetry (DSC), allows characterizing and determining various decomposition and degradation phenomena at specific temperatures, atmospheres, heating rates, and times. TGA analysis can be used to investigate the thermal stability, oxidative stabilities, as well as the compositional properties of the samples. The weight gain during TGA analysis is attributed to adsorption or oxidation, whereas weight loss is attributed to decomposition, desorption, dehydration, etc. (Ng et al. 2018). DSC is a technique used to investigate the response of polymers to heating. DSC can be used to study the melting of a crystalline polymer or the glass transition (Gómez et al. 2006). The thermal stability and decomposition temperature of the PHA and the biocomposites were determined by TGA, using Netzsch Jupiter F3 equipment. DSC spectrum analysis provided the thermal transition characteristics of the filaments (Fig. 13.5a). The first peaks for 100% PHA and 20% TMP fiber filaments were near 155–160 °C, and these values correspond to the melting point of the samples. The highest value was for the filament with the elastomer, and the temperature was 2 °C higher than that for 100% PHA, 20% fiber, and commercial filaments. The second peaks were between 276 and 290 °C and correspond to the single-step degradation reaction of polymer chains (100% PHA) or are related to the degradation of the hemicellulose fraction (in PHA-TMP biocomposites), resulting in organic acids, CO, CO_2, and condensable vapors (Cinelli et al. 2019). The third peaks were in the range of 335–350 °C for the composite with TMP fibers and commercial filaments and are assigned to cellulose degradation (Tsujiyama and Miyamori 2000). The presence of two degradation peaks for 100% PHA filament is due to the differences between copolymers in the polymer. Previous studies found that the degradation below 300 °C is via an unzipping reaction of the 4HB copolymer, whereas at temperatures above 300 °C, both the *cis*-elimination reaction of 4HB units and the formation of cyclic macromolecules of P(4HB) via intramolecular transesterification took place, in addition to the unzipping reaction (Chodak 2008).

TGA was performed on filaments to evaluate the thermal decomposition of the samples (Fig. 13.5b). The thermal degradation profiles of the filaments showed that most degradation events occur between 260 and 360 °C and were similar for all samples. The TGA profiles for wood composites usually show two degradation zones: a lower temperature for wood cellulose starting at about 250 °C (Jeske et al. 2012) and another zone at a higher temperature corresponding to the thermoplastic polymer. In this case, the degradation temperature of the PHA is similar to the degradation of cellulose. The onset degradation temperature for the PHA filament was 260.8 °C, and the thermal degradation increases to 6 °C (TMP fiber + PHA) and 10 °C (TMP fiber + PHA + elastomer) with the addition of fibers. The highest value of onset degradation was 274.2 °C for commercial filaments.

Finally, the residual mass measured at 800 °C was similar for the filaments 100% PHA (4.0%) and the composite with 20% TMP fiber (4.9% and 5.2%). The commercial filament showed a residual mass of 12.0%. Lignin is the most difficult component to decompose, as its degradation occurs slowly from the beginning of carbonization of other constituents up to 900 °C; the hemicelluloses and cellulose have completely degraded at 400 °C or less, but lignin requires more temperature to reach the complete degradation (Dias Júnior et al. 2019).

13.3.4 3D Printing

There are several procedures to manufacture structures by 3D printing. FDM or fused filament fabrication (FFF) is the most used (Le Duigou et al. 2019). FDM mostly uses thermoplastic polymeric inks. Numerous studies have been found on the use of thermoplastics to produce 3D structures, including the application of 100% thermoplastic polymers like PLA, epoxy resins, polyamide (PA), polycarbonate

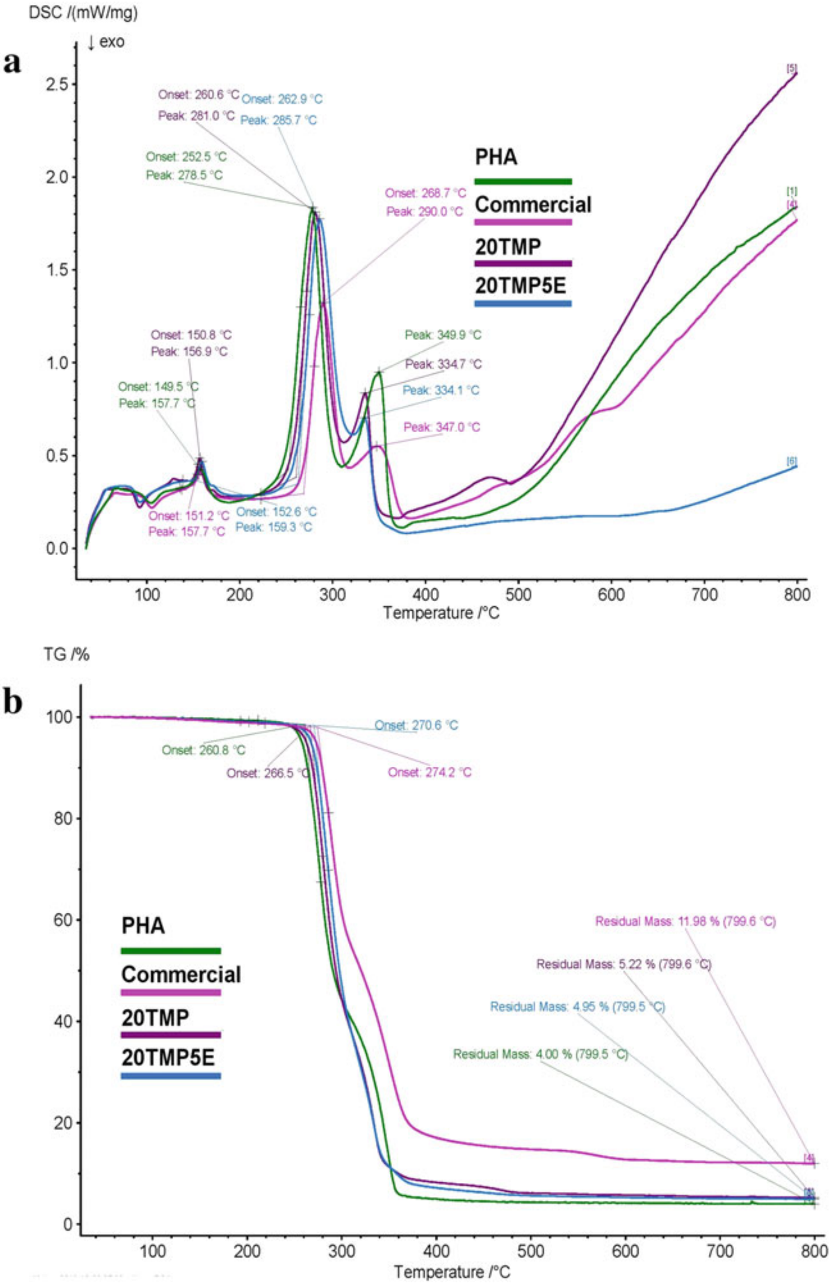

Fig. 13.5 (**a**) DSC and (**b**) TGA analysis of the studied samples

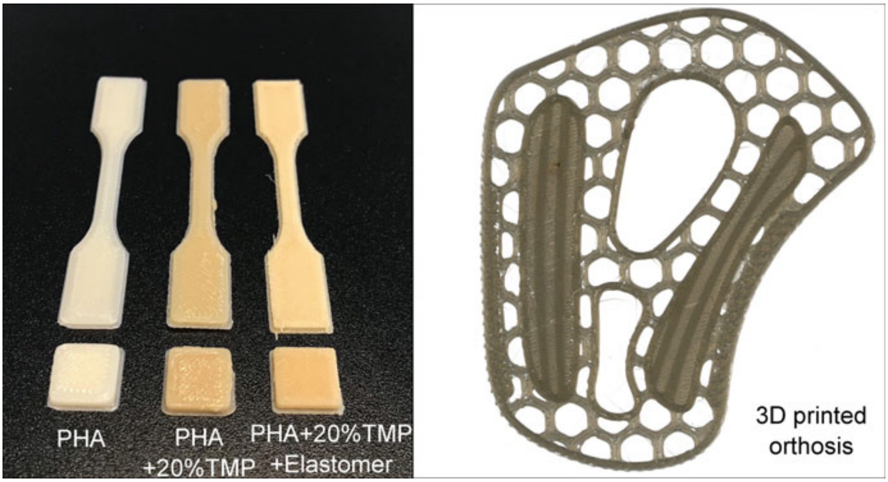

Fig. 13.6 3D structures obtained by FDM. (Left) 100% PHA, 20TMP, and 20TMP5E. (Right) 3D printed orthosis piece

(PC), and acrylonitrile butadiene styrene (ABS) (Wang et al. 2017) or composite inks. In recent years, biocomposite inks have gained attention due to the development of new materials such as particles (Nikzad et al. 2011; Isakov et al. 2016), fibers (Zhong et al. 2001; Ning et al. 2015), and nanomaterials (Fantino et al. 2016; Rymansaib et al. 2016), in combination with thermoplastic polymers. This has also been motivated by the increase in environmental awareness and the necessity of using renewable resources. The lignocellulosic biomass (wood and cellulose pulps) is mainly used as reinforcing material in matrices of thermoplastic polymers like polyethylene (Yamane et al. 1996; Kachrimanidou et al. 2014), PLA (Filgueira et al. 2017; Tao et al. 2017; Li et al. 2018), polyurethane (De Chen et al. 2019), or polypropylene (Wang et al. 2018; Zander et al. 2019).

In this study, the filaments of the commercial sample (wood flour composite, Table 13.1) were very fragile and broken during manipulation. Hence, the commercial sample could not be used for 3D printing. However, our 20TMP, 20TMP5E, and PHA filaments showed good flexibility and were used for 3D printing some structures and shapes (Fig. 13.6). A subsequent biodegradation analysis was performed with the square specimens shown in Fig. 13.6 (left). Additionally, 3D printed orthosis is exemplified in Fig. 13.6 (right). The use of 3D printing to produce structures focused on biomedical scaffolds and orthosis structures has been widely studied (Curodeau et al. 2000; Rodrigues et al. 2016; Zhu et al. 2016; Jammalamadaka and Tappa 2018; Maso and Cosmi 2019; Kangas et al. 2019). Acrylonitrile butadiene styrene (ABS) and polylactic acid (PLA) are the main materials used in rapid prototyping (Barrios-Muriel et al. 2020). However, a promising material for the production of biodegradable scaffolds is the PHA family. The interest in PHA polymers is due to additional properties like biodegradability and biocompatibility (Chiulan et al. 2018).

13.3.5 Analysis of Biodegradation in Soil

Recent work showed the micro- and macrostructure of biocomposites made of PHA and wood fibers after degradation in soil and compared to BioPE and PLA structures (Chan et al. 2019). The improvement in the biodegradation of PHA/fiber biocomposites as compared to other materials could be an advantage for its use in short-duration packaging and medical devices. Another alternative to increase the potential of the application of the biodegradable composites of PHA with fibers includes the use of simple, fast, and low-cost processing methods such as 3D printing. Weight loss is the usual physical method to assess biodegradation. In this study, the filaments and 3D printed structures were buried in soil under normal grass growth conditions. The samples were oven-dried at 80 °C for 1 h, weighed, and finally planted with grass seeds (Fig. 13.1). They were analyzed after 7, 12, and 23 weeks. 20TMP and 20TMP5E showed evidence of biodegradation, but it was not the case with the 100% PHA sample (Fig. 13.7a). The 20TMP5E sample exhibited the highest degradation, specifically in the center of the structure (Fig. 13.7b). On the contrary, the filaments did not show any degradation during the testing period, but they show the presence of fungi on the surface. The addition of TMP fibers increased the biodegradation in soil. The biocomposite samples showed the highest decrease in dry weight. The biocomposite sample containing 20% TMP and elastomer reached 8.0% of weight loss. The biocomposite sample containing 20% TMP only resulted in a weight loss of 4.7%. Finally, the 100% PHA structure reached 3.5% of weight loss after 23 weeks.

The degradation mechanism of PHA and PHA/wood fiber composites was previously described by Chan et al. (2019). The biodegradation is caused by enzymes from soil microorganisms. The biodegradation includes surface and bulk

Fig. 13.7 3D structures of PHA (**a**) and 20TMP5E (**b**) after the degradation test: top and cross view (bottom)

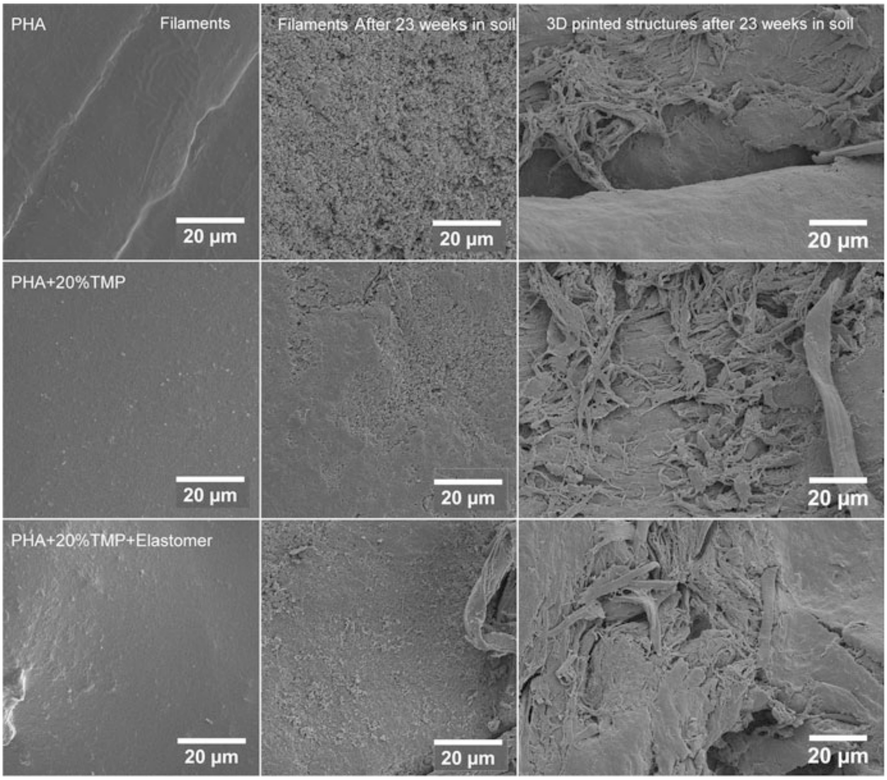

Fig. 13.8 SEM surface assessment of filaments and 3D structures. (Left column) Filaments before biodegradation study. (Middle column) Filaments after 23 weeks in soil. (Right column) 3D printed structures after 23 weeks in soil. All images were acquired at ×1000 magnification

erosion mechanisms. In surface erosion, the enzymes attack the structure, layer by layer, and the loss of weight is linear. In bulk erosion, it produces internal degradation, and the rate of degradation became exponential. Both mechanisms can be seen in the SEM photographs in Fig. 13.8. The structures showed some surface biodegradation. The surfaces exhibited micro-roughness and some fibers appeared on the surface. Figure 13.8 (right) shows the SEM surface assessment of 3D printed structures. The images exemplify the microstructure of the surface of the planted squares. The filaments (Fig. 13.8, left) were subjected to the same biodegradation conditions as the corresponding 3D printed square structures and showed some surface biodegradation (Fig. 13.8, middle). However, unlike the 3D printed squares, the weight loss in filaments was negligible after 23 weeks. The results showed that the biodegradation rate of the samples with 20% of TMP fibers and 80% PHA increased 12% in soil, compared to those of 100% PHA (from 0.055 to 0.062 mg day^{-1} cm^{-2}). The inclusion of elastomer in the formulation caused an increase of 73% (increase up to 0.096 mg day^{-1} cm^{-2}). However, the determination of the rate of biodegradation using only weight loss is less sensitive to all the phases

Table 13.3 Comparison between the weight losses in biodegradation studies in soil

Composites	Depth in soil (cm)	Period (month)	Zone	Weight loss (%)	Refs.
70 × 15 × 1.6 mm (100% PHBV)	7	12	Queensland, Australia (27.5°S, 152.9°E)	2	Chan et al. (2019)
70 × 15 × 1.6 mm (80% PHBV, 20% wood flour)				6.7	
70 × 15 × 1.6 mm (50% PHBV, 50% wood flour)				12.7	
70 × 15 × 1.6 mm (50% PLA, 50% wood flour)				3.7	
Pellets 100% PHB (10 mm × 2.5 mm)	15	10	An Giang, Vietnam (22°45′N, 105°48′E)	55.0	Boyandin et al. (2013)
Pellets 100% PHBV (10 mm × 2.5 mm)				35.0	
Pellets 100% PHB (10 mm × 2.5 mm)			Nha Trang, Vietnam (12°14′N, 109°11′E)	28.0	
Pellets 100% PHBV (10 mm × 2.5 mm)				8.0	
3D structures 20% fiber wood, 80% PHA (2.56 cm^2)	5	5	Posadas, Argentina (27°21’S, 55°53’W)	4.7	*Present work*
3D structures 20% fiber wood, 80% PHA + elastomer (2.56 cm^2)				8.0	
3D structures 100% PHA (2.56 cm^2)				3.5	

passed by the material to the environment. This can be completed by combining physical analysis, such as weight loss with standardized respirometric methods (Dilkes-Hoffman et al. 2019). The weight losses of samples analyzed in different places worldwide are shown in Table 13.3. Our values of weight loss are similar to those obtained by Chan et al. (2019) using 20% of wood flour and 80% PHBV, but the degradation time is lower. This can be because the humidity and temperature in the period of the study were relatively high (values higher than 20 °C in average temperature and atmospheric humidity higher than 70% in periods that include winter and part of spring in Posadas, Misiones). On the other hand, the values in weight loss reached by Boyandin et al. (2013) were the highest. The difference is that the climate in the places where the studies were performed are tropical zones and the period of the study included the Monsoon with precipitation up to 3000 mm, while in Queensland and Posadas, the mm of the fallen rain was 890 and 503 mm.

13.4 Conclusion

Nowadays, legislation aspects and production costs are factors that affect the demand for PHAs by the market. Additionally, the current studies show promising markets for PHA polymers (Choi and Lee 1999; European Bioplastics 2019). The applications are related to the additional properties such as biodegradation and biocompatibility of the material and involve medical products: drug delivery by conjugation (Zawidlak-Wegrzyńska et al. 2010) or encapsulation (Wang et al. 2019), orthosis or scaffold parts and tissue engineering (Rathbone et al. 2010; Michalak et al. 2017; Elmowafy et al. 2019), cosmetic additives (Maksymiak et al. 2015), and bioplastics with degradation in short times (Fig. 13.9). Films from PHA materials have gained attention to be applied in food packaging due to the renewability, biodegradability, and water vapor barrier properties of the polymer: PHB acts as a better light barrier in the visible-UV regions than PLA and PHBV in blow-molded bottles and as a coating on paper (Khosravi-Darani and Bucci 2015). The studies include the use of PHAs in combination with inorganic particles, cellulosic fibers, plasticizers, PLA, or nanoparticles to improve properties (gas barrier, antimicrobial, thermal behavior, mechanical properties, hydrophobicity, etc.) (Keskin et al. 2017). Currently, PHAs are expensive polymers. However, it is expected that optimized production technology, environmental concerns, and societal awareness will lead to a higher demand for biobased polymers, higher production capacity, better

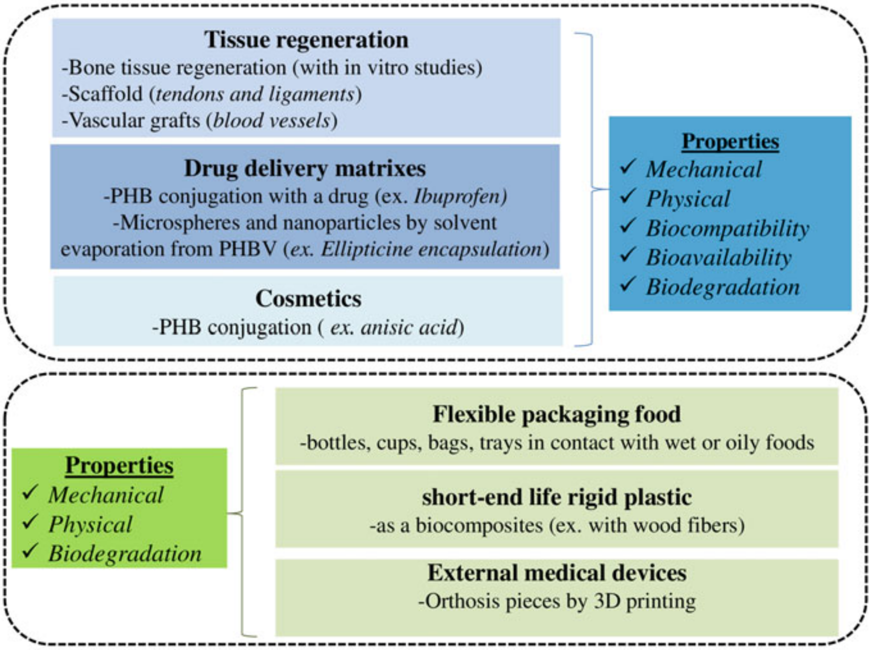

Fig. 13.9 Summary of the promising applications of PHAs

availability, and thus lower prices. This will also be facilitated by more favorable regulations and directives advised by stakeholders and imposed by decision-makers.

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14 Bio-Based Bioplastics in Active Food Packaging

Elena Stoleru, Anamaria Irimia, and Elena Butnaru

Abstract

Plastics are the most common packaging materials and bioplastics (in particular biologically derived plastics) proved that they can be used in active food packaging applications, mainly in applications that include products with short shelf-life. The bio-based bioplastics are split in two categories, namely, biodegradable and non-biodegradable materials. Relatively recent is evidenced that bioplastics can represent a solution to overcome the drawbacks associated with the conventional plastics used in food packaging (environmental issues, health problems, etc.). Packaging plays an important role to maintain food quality and active packaging appears to be a smart solution to successfully extend shelf-life or to enhance food quality and safety. This chapter aims to review the latest developments and challenges in the field of bio-derived plastics applied in food packaging sector (focusing on active food packaging). It highlights the bio-derived plastics most used in this field and their advantages and limitations over common plastics and gives an overview of the recent developments in active food packaging applications.

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Keywords

Bio-based bioplastics · Food packaging · Biodegradable · Food quality · Applications

14.1 Introduction

Starting from the middle of the last century, the progress in production of fossil-based plastics bestows on significant economic expansion and meaningfully contributed to human welfare. However, the facts that the most of the plastic products are packaging and single-used items, with very short service life before disposal, and scarcely recycling (18% of plastics waste) and incineration have led to plastic pollution. Arose stringent health concerns associated with the plastic pollution and the global warming has increased the demand for alternative materials (Jem and Tan 2020). With the aim to overcome the issues associated with the traditional petroleum-based plastics, the bio-plastics have gained special attention. At this time the word "bioplastics" is yet used in a confusing manner. There is a widespread wrong assumption that if something has biological origin (e.g. biomass), therefore it must be also biodegradable. In any case, not all products that are derived from biological resources are inherently biodegradable. It is important to emphasize and understand that bio-based plastics are not always biodegradable and that biodegradable plastics are not always bio-based (Rujnić-Sokele and Pilipović 2017). Based on the statements of European Bioplastics organization the bioplastics can be defined as plastics based on renewable resources (bio-based) or as plastics which are biodegradable and/or compostable polymers or both (Rahman et al. 2019). It is illustrated in Fig. 14.1 a schematized classification of bioplastics compared with the conventional plastics (fossil-based non-biodegradable). The biodegradation capacity of a material is dependent especially on its tchemical structure and not necessarily on its resource type. In an explicit way to say, 100% bio-based plastics may be non-biodegradable, and 100% fossil-based plastics can biodegrade (European Bioplastics 2018). The increasing demand for healthier and more nutritional food, containing reduced amount of preservatives without compromising human and environmental safety, needs the development of new conservation and packaging technologies. The elaborated conservation strategies include active packaging (AP), non-thermal treatments (high pressure, pulsed electric fields), modified atmosphere packaging, etc. These new strategies have received a considerable attention for the last 10–20 years, especially AP (Raouche et al. 2011). AP attempts to strengthen beneficial interactions in the food/packaging/environment system to improve healthiness and quality of the packaged food and increase its shelf-life (Catalá et al. 2015). As defined in the European regulation (EC) No 450/2009, active packaging systems are designed to *deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food* (Yildirim et al. 2018).

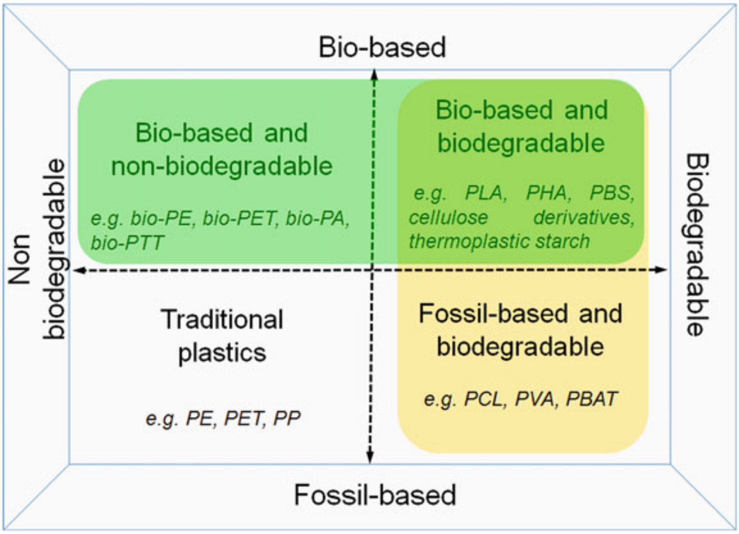


Fig. 14.1 Schematic representation of bio-plastics and conventional plastics (Ilyas and Sapuan 2020)

The wide diversity of **AP systems** comprises additives with a multitude of active functions, namely, absorbing/scavenging properties, releasing/emitting properties, removing properties, and temperature, microbial, and quality control (Vilela et al. 2018). These AP materials could be obtained by the incorporation of active compounds into already available packaging substrates (with role of matrices) or by the application of coatings with specific functionality through physical or chemical surface modification (Vasile 2018). This chapter gives an overview of the main bio-derived plastics, their properties, most common limitations and recently reported applications in active food packaging. It brings into focus only the biologically derived bioplastics, both biodegradable and non-degradable; the other class of bioplastics (fossil-based bioplastics) represents the subject of another book chapter.

14.2 Biologically Derived Biodegradable Plastics

According to European Bioplastics Association, the term “bio-based” is defined as *a material or product that is (partly) derived from biomass (plants)*. This group of materials includes plastics that derive both from non-petroleum biological resources and are in the same time biodegradable in natural environments (European Bioplastics 2018). Over time, **biodegradation** was defined in a simplistic way, namely, the breakdown of organic matter by microorganisms. Through this definition is emphasized, in particular, the biological activity of this phenomenon. As time went on, it was revealed that the abiotic factors cannot be excluded from the biodegradation process. Nowadays, it is established that the decomposition of organic matter includes a synergistic action of biotic and abiotic factors. Moreover, biodegradation of polymeric materials includes several steps and the process can stop at each stage (Lucas et al. 2008). This class of bioplastics comprises various polymers such as poly(lactic acid) (PLA), poly(hydroxyalkanoates) (PHAs), thermoplastic starch, and blends made of thermoplastically modified starch and cellulose materials (regenerate cellulose or cellulose acetate (CA) with low degree of substitution) (Iwata 2015; Thakur et al. 2018). Unlike cellulose materials, they started to be available on an industrial scale only for the last couple decades.

14.2.1 Poly(Lactic Acid)

14.2.1.1 Structure and Obtaining Methods

Poly(lactic acid) (PLA) is a biodegradable thermoplastic derived from lactic acid, which is the most widely occurring carboxylic acid in nature, generally originated from corn, sugar cane, or other plant residues (Ahmed and Varshney 2011). There are two main methods, illustrated in Fig. 14.2, to produce lactic acid, namely: bacterial fermentation of carbohydrates and chemical synthesis (Kricheldorf et al. 1996). The mainly synthesis routes of PLA are the polycondensation of lactic acid (2-hydroxy propionic acid) and/or ring-opening polymerization of lactide (Castro-Aguirre et al. 2016). The industrial production of PLA involves in general bacterial

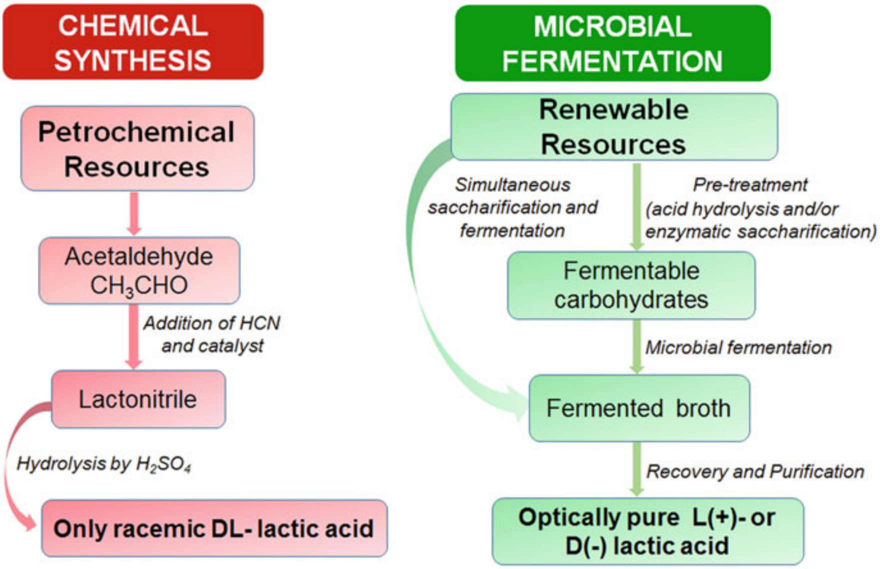


Fig. 14.2 Production methods of lactic acid by chemical and microbial fermentation (Kumar et al. 2020; Wee et al. 2006)

fermentation method mainly because the chemical synthesis method is not economically feasible. The bacterial fermentation route is also preferred by the major producers, namely, NatureWorks LLC and Corbion® (Hartmann 1998). Lactic acid has two optical isomers: *dextrorotatory* form called D(+) or R lactic acid, which is the natural and biologically important isomer, and *levorotatory* form called L(−) or S lactic acid that can be produced by microorganisms or by racemization (Ahmed and Varshney 2011). PLA with high content of L-form isomer is highly crystalline, and its biodegradability depends on the content of D-form isomer (Datta et al. 1995).

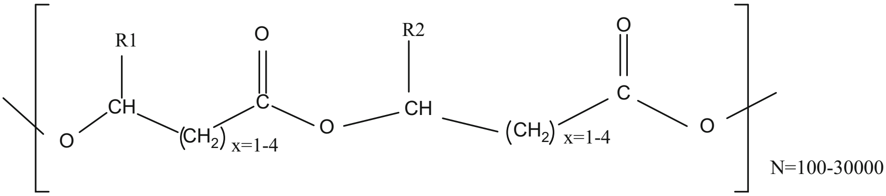
14.2.1.2 Properties and Limitations

PLA is a polyester with thermoplastic properties that can be processed by spinning into fibers and stretching into rigid films, extruded into sheets, and injected into molds (Jem and Tan 2020). The PLA features that have attracted a lot of attention in food packaging domain are easy processability, transparency, printability, as well as biodegradability in compost, large-scale production, high-tensile strength, and reasonable barrier properties for some applications (Kalia and Averous 2011). When compared to PET and polyolefins its main drawbacks are the low thermal resistance, excessive rigidity, high brittleness with less than 10% elongation at break, and high permeable properties (Khosravi-Darani and Bucci 2015). The high rigidity of PLA could be overcome by incorporation of plasticizers (Rapa et al. 2017), but this leads to a decrease in oxygen barrier and transparency. Moreover, PLA’s slow degradation rate and its high cost (2.8 times more expensive than polypropylene and 3.2 times more expensive than high-density polyethylene) are other important shortcomings, which still limit to large-scale application as packaging material (Stoleru et al. 2016a; Hegde et al. 2019; Claro et al. 2016).

Worth to mention is that even if some features of PLA can be a disadvantage in a particular application, in another kind can be an advantage. For example, the low water vapour barrier of the bio-based PLA is a shortcoming for a water bottle but a benefit in packaging of vegetables and fruits, where higher permeability is necessary (van den Oever et al. 2017). With the continually advancement of the research in this direction, potential routes to address the limitations of PLA were found, such as copolymerization, blending, incorporation of plasticizers, or the addition of reinforcing phases (fibers or fillers and additives) (Yuan et al. 2013; Siakeng et al. 2020; Sharma et al. 2020; Darie et al. 2014).

14.2.2 Poly(hydroxyalkanoates)

Poly(hydroxyalkanoates) (PHAs) are poly(esters) obtained by polymerization of R-hydroxyalkanoic acid monomers, which are produced in nature by many microorganisms, including Gram-positive and Gram-negative bacteria, and present thermoplastic or elastomeric features. PHAs are garnered as cytoplasmic granules, reaching accumulation percentages of up to 90% of the dry mass, as intracellular carbon and energy storage compounds (Keshavarz and Roy 2010; Madison and



Scheme 14.1 General structure of poly(hydroxyalkanoates). R1 and R2 are alkyl groups (C1–C13) (Keshavarz and Roy 2010; Anderson and Dawes 1990)

Huisman 1999; Anderson and Dawes 1990; Lee 1996). PHAs are the most versatile fully biodegradable polymers with properties similar to conventional plastics (Keshavarz and Roy 2010; Steinbuchel and Fuchtenbusch 1998). In addition to being biodegradable, PHAs can also be recycled like conventional thermoplastic (fossil-based) materials (Madison and Huisman 1999). First discovered (in 1920s by the French microbiologist Maurice Lemoigne) and most common polyester belonging to PHAs class was the biopolymer poly(hydroxybutyrate), P(3HB). P(3HB) was found to be formed in intracellular granules in *Bacillus megaterium*, a Gram-positive bacterium (Lemoigne 1926; Keshavarz and Roy 2010). Among this group of polymers, P(3HB) was the most studied and has gained the attention in the commercial sector (Madison and Huisman 1999).

14.2.2.1 Structure of PHAs

The numerous polymers from PHA class that have been identified so far are polyesters based on 3-hydroxy fatty acid monomers, mainly linear, with head-to-tail configuration and with the general formula presented in Scheme 14.1. These polymers are classified according to the number of carbon atoms ranging from 4 to 14. Moreover, depending on the type of monomeric units can result homopolymers or heteropolymers (Keshavarz and Roy 2010). In general, the PHAs are sterospecific, with the carbon atom substituted by hydroxyl in *R*(−) configuration, except in cases where there is no chirality (Madison and Huisman 1999).

14.2.2.2 Properties and Limitations

The class of PHAs includes materials with various and different mechanical properties ranging between strong crystalline to elastic (Anjum et al. 2016). The poly(hydroxyalkanoates) with short chain length are rigid and brittle and have high crystallinity (between 60 and 80%), while medium chain length PHAs and their copolymers present flexibility and elasticity (with elongation at break ranging between 300 and 450%), low degree of crystallinity (20–40%), lower melting temperatures, and glass transition temperatures (T_g) below room temperature (Ragaert et al. 2019).

Native P(3HB) presents high brittleness and stiffness. This lack of flexibility narrows its interval of applications (Braunegg et al. 1998). In the line of applicability, the copolymers gathered a particular attention, mainly the copolymers of 3HB

Table 14.1 Physical properties of some representative PHAs, PLA, and conventional polyolefins

Plastic	Parameter				References
	T_m (°C)	T_g (°C)	Crystallinity (%)	Elongation at break (%)	
P(3HB)	177	2	70	5	Madison and Huisman (1999)
P (3HB-3 HV)	145	-1	56	50	Madison and Huisman (1999)
P (3HB-4HB)	150	−7	45	444	Madison and Huisman (1999)
P (3HO-3HH)	61	−36	30	300	Madison and Huisman (1999)
PLA	140–180	57–58	10–12	5	Jem et al. (2010), Stoleru et al. (2020)
PP	176	−10	60	400	Madison and Huisman (1999)
LDPE	109	−125	25–50	450–810	Jordan et al. (2016), Polymer Properties Database (2019a)
HDPE	134	−110	60–80	600–1350	Jordan et al. (2016), Brown et al. (2007), Polymer Properties Database (2019b), Omnexus (2020)

T_m is melting temperature; T_g is glass transition temperature
P(3HB) poly(3-hydroxybutyrate), *P(3HB-3 HV)* poly(3-hydroxybutyrate-co-3-hydroxyvalerate) containing 20% 3 HV, *P(3HB-4HB)* poly(3-hydroxybutyrate-co-4-hydroxybutyrate) containing 16% 4HB, *P(3HO-3HH)* poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) containing 11% 3HH, PLA is poly(lactic acid), PP is poly(propylene), LDPE is low-density poly(ethylene), and HDPE is high-density poly(ethylene)

and 3HV (e.g. P(3HB-co-3HV)s), especially because they have much lower melting points, and are less crystalline, more ductile, easier to mold, and tougher, than pure P (3HB) (Luzier 1992). The advantages of copolymers make them attractive possibilities for commodity items. Variation in the copolymers of the content of 3HV result in obtaining materials with different properties, covering a wide range of thermomechanical features. At the moment are produced various copolymers P (3HB-co-3HV), with 3HV content ranging between 0 and 24 mol%, using *Ralstonia eutropha* bacterium and commercialized by Monsanto under the trademark BIOPOL® (Braunegg et al. 1998). Among the bioplastics polyesters (PHAs and PLA) there are various polymers with characteristics similar to conventional fossil-based plastics as revealed in by data listed in Table 14.1.

14.2.3 Cellulose and Derivatives

Native cellulose is scarcely used in food packaging applications due to its strong hydrophilic nature, pure solubility, and high crystallinity. Hence, cellulose derivatives or blends/composites with synthetic polymers are preferred in this area of application (Rahman et al. 2019; Sdrobis et al. 2012; Irimia et al. 2017).

14.2.3.1 Cellophane Films

Cellulose can be treated with chemicals like NaOH, H_2SO_4, CS_2, etc. to produce cellophane that presents excellent mechanical characteristics (Rahman et al. 2019). Cellophane films—which technically are not plastics—are highly transparent, rather stiff, and made up of short cellulose fragments that can be chemically modified to make them soluble and malleable. The material cannot be melted or deformed under heat and pressure due to the high number of hydroxyl groups and the resulting presence of hydrogen bonds, and this implies that the films have an excellent dimensional stability (van den Oever et al. 2017).

14.2.3.2 Cellulose Acetate (CA)

Cellulose acetate (CA), on the other hand, is heat sealable and much more stable with regard to moisture due to acetyl groups and the resulting reduced number of hydrogen bonds and plasticizers. However, the barrier properties are lower compared to cellophane (Peelman et al. 2013; Krauter et al. 2019).

14.2.3.3 Carboxymethyl Cellulose

Carboxymethyl cellulose (CMC) is considered the most adequate option for production of biodegradable films. CMC has high crystallinity, is highly soluble in water, and presents film-forming ability. The CMC films are transparent, easy processable, non-allergenic, and non-toxic and have affordable price. It has been largely used in the food industry, in various bio-medical applications, in detergents production, in oil drilling, in paper industry, and in textile printing (Al-Tayyar et al. 2020). Various environmental friendly film-forming materials were developed by using modified fibers of wood cellulose, like α-hydroxysulfonic acid cellulose (HSAC), taurine cellulose, or dicarboxylic acid cellulose. The resulted HSAC film presented outstanding mechanical properties and manifested improved water vapour barrier properties comparing with cellophane (Sirviö et al. 2013). There have been prepared biodegradable film composites by Rydz et al. (2018) using modified cellulose fiber as reinforcing agents for polyvinyl alcohol (PVA). The study pointed out that the addition of modified cellulose had led to an increase of tensile strength of the composite films and is proposed as potential packaging material (Rydz et al. 2018). Indumathi et al. (2019) have reported the obtaining and characterization of chitosan-cellulose acetate phthalate films incorporated with ZnO nanoparticles (CS-CAP-ZnO nano) through solvent casting method. They observed that the content of nano ZnO had influenced the thermal stability and barrier properties of the obtained films, namely, these properties increased directly proportional with the amount of the ZnO (5% w/w being the optimal). The CS-CAP film loaded with 5%

ZnO presented the most suitable mechanical properties for food packaging application.

14.2.3.4 Bacterial Cellulose (BC)

Bacterial cellulose (BC) is mainly produced through microbial fermentation performed in static or agitated cultures (Stoica-Guzun et al. 2013). BC has gained substantial attention to be applied in food packaging due to its fine properties, such as high purity of cellulose content and refined nanofibrous network. Generally, in this domain it is used as a proper carrier for various antibacterial agents. In particular, BC obtained by *Acetobacter xylinum* fermentation is widely used in food processing due to the improved properties compared to plant cellulose (higher water holding capacity, tensile strength and fiber content, softer texture) (Gao et al. 2014).

Bacterial cellulose fibers can be used in combination with PVA, in order to obtain packaging materials, with improved properties like mechanical strength and better biodegradability. Unfortunately, because of the high moisture sensitivity, the application of BC/PVA in food packaging is limited (Stoica-Guzun et al. 2011). BC was also used in nanocomposite materials preparation with poly(ε-caprolactone) (PCL). The nanocomposites were obtained in situ through supplementation of the bacterial cellulose culture medium with adjustable amounts of PCL powder. The BC/PCL films were produced by hot pressing the polymeric blends obtained after incubation. The obtained materials had fine thermal and enhanced mechanical properties compared with PCL, improved biodegradability, and biocompatibility, which makes these materials suitable for food packaging application (Figueiredo et al. 2015).

14.2.4 Thermoplastic Starch (TPS)

14.2.4.1 Properties and Limitations of TPS

In the field of renewable and sustainable plastics, **starch** is intensively used either as a feedstock or as a component because of its low cost, wide availability, and biodegradability.

Starch $(C_6H_{10}O_5)_n$ is composed of carbon, hydrogen, and oxygen, in a mixture of 18–28% amylose and 70–85% amylopectin (Khan et al. 2016). Starch is a semicrystalline polymer with crystallinity about 20–45%; the crystallinity degree is correlated to the amylose content or to amylopectin chain length (amylopectin with long chains forms a strong crystalline structure) (Cornejo-Ramírez et al. 2018). Native starch is not suitable for food packaging applications because it is brittle and semi-permeable to carbon dioxide, moisture, oxygen, lipids, and flavour components (Shah et al. 2015). It has poor thermal processing stability because it starts to decompose around 220 °C, before melting. In order to use starch as a food packaging material it has to be converted into thermoplastic by heating the polymer in the presence of water, glycerol, glucose, sorbitol, urea, and ethylene glycol as plasticizers (Ismail et al. 2016; Abera 2020). Most common methods used for obtaining thermoplastic starch are extrusion and solvent casting method. TPS can be successfully used in food packaging because it is relatively cheap (market and consumer needs), abundant, and biodegradable. However, TPS has some limitations; it has poor mechanical properties and it is sensitive to moisture content. Generally, the starch content in starch blends can vary from 5 to 90 wt% (Bátori et al. 2018). Blending starch with non-biodegradable polymers including LDPE (Chatkitanan and Harnkarnsujarit 2020) or biodegradable materials, such as PLA (Collazo-Bigliardi et al. 2019), PVA (Domene-López et al. 2018), or chitosan (Castillo et al. 2017), results in obtaining materials with low cost and improved water barrier, enhanced processing, and mechanical properties (Yu and Christie 2005). In general, blending TPS with PVA is preferred in order to enhance the mechanical strength and water resistance and also to reduce environmental pollution. However, these packaging blends are likely unacceptable from the economic viewpoint due to the additional high processing cost in comparison to commercial plastic packaging materials. Domene-López et al. (2018) investigated the mechanical properties of potato starch/PVA/rosin blends obtained by melt mixing and compression molding. It was found that the addition of 8% rosin to starch/PVA blends led to improved mechanical properties (tensile strength values >10 MPa; elongation at break ~2000%) comparable to those offered by polymers such as LDPE. Environmentally friendly packaging materials based on rice starch and PVA were synthesized by casting method by Parvin et al. (2010), and the obtained packaging materials were proposed to be used as shopping and garbage bags.

14.2.5 End-of-Life Options of Bio-Based Biodegradable Plastics

Biodegradability represents an end-of-life possibility that involves exploiting the capacity of microorganisms, which are found in a particular environment, to completely eliminate the disposed plastic products. This can be achieved in a timely, safe, and efficacious approach via the microbial food chain (Narayan 2009; Rujnić-Sokele and Pilipović 2017). The biodegradability of plastics is influenced by numerous factors such as the type of raw materials, the chemical composition and structure of the final product, as well as on the environment under which the product is expected to biodegrade (Rujnić-Sokele and Pilipović 2017). It represents the capacity of microorganisms to use a polymer as a sole source of carbon and energy. The mechanism of polymer biodegradation depends both on its structure and properties (molecular weight, crystallinity, hydrophobicity, the presence of functional groups, use of additives, plasticizers) and on the environmental conditions (temperature, moisture, oxygen, pH). The usual mechanism is hydrolysis, in which cleavage of the ester bonds leads to polymer chain scission yielding low molecular weight fragments (Kale et al. 2007).

14.2.5.1 Biodegradability of PLA and PHAs

Particularly, the degradation of PLA happens mostly through scission of ester bonds, biologically resulting to lactic acid, which is a product of the carbohydrate metabolism (Cong et al. 2012). PLA can biodegrade both in aerobic and anaerobic conditions (Bubpachat et al. 2018). The PLA biodegradation process and duration heavily depend on the environmental factors such as heat, humidity, pH, oxygen, microbes, and so on. Native PLA decays best in environments with elevated temperatures (around 60 °C, its glass transition temperature), because rapid chemical hydrolysis occurs, and which have a rich presence of microorganisms. But even in such conditions, PLA will start to show signs of biodegradation approximately in 6 months (Bohlooli 2019). Such temperature is not reached in normal soil conditions, only in industrial composting environment (Stoleru et al. 2020). Nevertheless, an important feature of PLA is that after its biodegradation will not pollute the environment (Qi et al. 2017). Compared to other biodegradable plastics (e.g. PCL or PHB), PLA materials are less sensitive to attack of microbial in the native soil environment, mainly because PLA-degrading microorganisms are not widely distributed in natural soils and present at very low percentages (Decorosi et al. 2019).

Numerous and various microorganisms (bacteria and fungi) are able to degrade PHAs. This property is dependent on both their ability to secrete specific extracellular PHA depolymerases, namely carboxyesterases, and physical state of the polymer (amorphous or crystalline) (Jendrossek and Handrick 2002).

14.2.5.2 Biodegradation of Cellulose-Based Materials

The degree of substitution (DS) is an important factor in determining susceptibility of soluble cellulose derivatives to biological degradation. Although cellulose acetate (CA) can be produced with wide range of DS, due to good solubility in various solvents, molecular weights, and melt properties, most common is DS = 2.5. CA is prepared by acetylating cellulose, which is readily biodegraded by organisms that utilize cellulase enzymes. Due to the additional acetyl groups, CA requires the presence of esterases for the first step in biodegradation. After partial deacetylation by enzymes, or by partial chemical hydrolysis, the polymer's cellulose backbone is readily biodegraded. CA can also be photochemically degraded by UV wavelengths shorter than 280 nm, but has limited photodegradability in sunlight due to the lack of chromophores. Photodegradability can be significantly enhanced by the addition of titanium dioxide that causes surface pitting, thus increasing a material's surface area that enhances biodegradation. The combination of bio- and photodegradation can enhance the overall degradation rate (Puls et al. 2011). In general, cellulose and cellulose derivatives are used in various polymeric blend or composite materials to enhance the biodegradability process. For instance, CA and cellulose acetate phthalate (CAP) were blended with poly(methyl methacrylate) (PMMA) (PMMA-CA and PMMA-CAP) to obtain biodegradable films. Biodegradability of these blends has been studied by weight loss method, using four different methods: enzymatic degradation, soil burial test, degradation in phosphate buffer, and activated sludge degradation followed by water absorption tests to support the degradation studies. The results showed that PMMA forms miscible blends with either CA and CAP in the entire composition range, probably due to the formation of hydrogen bond between the carbonyl group of PMMA and the free hydroxyl group of CA and CAP. Also it was demonstrated that polymeric blend films of PMMA-CA and PMMA-CAP are biodegradable in all tested conditions and that the biodegradability increases with the increase in CA and CAP content in the blend compositions. At the same time, a higher content of CA or CAP (more than 45%) can cause a decrease of some important PMMA properties (Bhat and Kumar 2006).

14.2.5.3 Biodegradation of TPS-Based Materials

The degradation of starch can readily and completely be performed in aerobic (use oxygen and produce CO_2 and water) or anaerobic conditions (strictly dependent on temperature). An advantage of obtaining polymeric blends or composites containing starch is to promote a faster biodegradation rate of various synthetic polymers (e.g. PVA, PCL) (Mohee et al. 2008; Nunziato et al. 2018; Cuevas-Carballo et al. 2017). Namely, such behavior was noticed by Mohee et al. (2008) studying the biodegradability of Novamont Mater-Bi (composed of 60% starch and starch derivatives and of approximately 40% synthetic resin that is hydrophilic and biodegradable) under aerobic environment followed by anaerobic conditions. Also, Russo et al. (2009) concluded that a higher quantity of starch in thermoplastic starch/PVA blends resulted in increased biogas production and fastened the rate of blend degradation in anaerobic conditions.

It is well known that the incorporation of natural active compounds into biodegradable films is an excellent choice to extend the shelf-life and maintain the food quality. However, it is important to know how these active substances could affect the biodegradation behavior of the active packaging material. The effect of eggshell powder and citric acid on the TPS biodegradation was investigated by Prapruddivongs and Wongpreedee (2020). Based on the biodegradation test results, it was observed that after soil burial for 30 days, the TPS materials without eggshell powder showed erosion over almost the entire surface, while the surface of samples containing eggshell powder remained unchanged. The authors concluded that the eggshell powder could act as a hydrolytic retardant for citric acid-filled thermoplastic starch through calcium citrate formation. The effect of antimicrobial substance incorporation into starch-PVA blend films on their biodegradation under controlled composting conditions was evaluated by Cano et al. (2016). The test was based on the measurement of the CO_2 generated in the process, which was considered proportional to the percentage of biodegradation. The results indicated that the antimicrobials affected the biodegradation profile of the starch/PVA films; neem and oregano essential oils had a very small influence on the maximum biodegradation rate (5–7%/day, after 9–12 days), while the silver nanoparticles completely changed the biodegradation profile, reaching the maximum biodegradation rate (about 15%/day) after 6 days of composting exposure.

14.2.6 Applications of Bio-Derived Biodegradable Plastics in Active Food Packaging

14.2.6.1 PLA- and PHA-Based Active Packaging Materials

Currently, PLA is the most commercially used bio-polyester in food packaging applications (Khosravi-Darani and Bucci 2015). Due to its intrinsic properties, PLA can be a convenient material in developing sustainable active food packaging.

In this field, PLA could be used as a matrix or carrier for active agents providing a controlled and sustained release system, where the slow release of the active agents from the PLA polymer to the wrapped/packed food could offer a long-term protection against food spoilage (Heydari-Majd et al. 2019). The active agents used for AP are following the same trend as the packaging materials toward natural-based and eco-friendly alternatives. Natural additives added into polymeric matrices (e.g. PLA) can have various roles such as reinforcing, antioxidant, or antimicrobial agents determining enhanced barrier and wetting properties of the packaging material (Papadopoulou et al. 2019; Sebastien et al. 2006; Del Nobile et al. 2009; Stoleru et al. 2016b). Lately, herb extracts and essential oils become attractive bioactive agents for food packaging applications. Numerous researches are reported in the domain literature (Heydari-Majd et al. 2019; Butnaru et al. 2019; Darie-Nita et al. 2018; Vasile et al. 2017; Romani et al. 2017; Irimia et al. 2017). In a research performed by Miao et al. (2019), the addition of fennel (FEN) oil into biodegradable films based on PLA and PHB rendered to the base material antimicrobial activity and were successfully tested for oysters preservation. The study revealed that shelf-life of oysters could prolong within 2–3 days, while maintaining their quality, when packed in PLA-PHB-FEN film (Miao et al. 2019). In another study, films based on PLA and P3,4HB blends (obtained by melt blending) loaded with ginger (GEO) and angelica (AEO) essential oils were used for peach preservation. The aging and spoilage of the peach was traced visually through the Low-Field Nuclear Magnetic Resonance (LF-NMR) method. The color of the proton density map of the PLA-P3,4HB-AEO group was light blue, and the preservation effect was the best—Fig. 14.3. The film added with AEO showed the best preservation action, effectively delaying the oxidation of peach, maintained the combined water, and extended the shelf-life of peaches to more than 15 days (Jiang et al. 2020).

Active PLA-based films were developed by Safaei and Azad (2020) through incorporation of different amounts of propolis extract, as active agent, and tested on dry meat sausage. Further incorporation of PEG/$CaCO_3$ into PLA/propolis extract films remarkably improved the antimicrobial activity of films, enhanced flexibility and stiffness of polymers, and reduced their tensile strength.

14.2.6.2 Celullose Derivatives in Active Food Packaging Materials

Cellophane films can be colored and are well known as candy wrappings. Other applications include luxury films for flower wrappings and the glossy transparent films that cover tea boxes. The biodegradable (barrier) films are available in a wide range of grades, and they can be used to pack products ranging from cheese to coffee and chocolate. Because of the relatively high costs, cellophane is usually coated with other plastics or even replaced by them (van den Oever et al. 2017). Cellulose acetate is extensively applied as food packaging for fresh products and baked goods (Pawar and Purwar 2013). Rodríguez et al. (2014) have proved that nanocomposites based on CA, organically modified montmorillonite (Cloisite 30B), thymol as a natural antimicrobial component, and triethyl citrate as a plasticizer are really promising materials in food-packaging application. In a study by De Moura et al. (2012) was revealed that HPMC and silver nanoparticles' (AgNPs) composite films can be

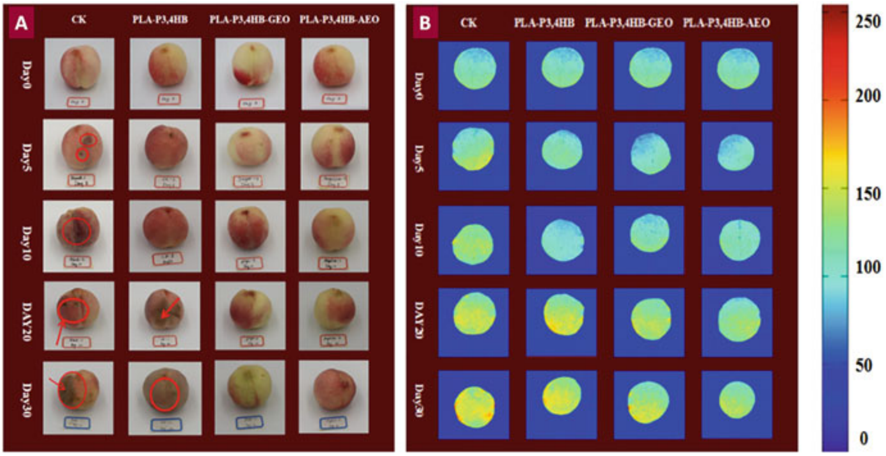


Fig. 14.3 Physical map (**a**) and proton density image (**b**) of peaches during storage in different active films based on PLA-P3,4HB incorporated with ginger (GEO) and angelica (AEO) essential oils; CK represents the group without film packaging (Adapted from Jiang et al. 2020; Copyright Elsevier 2020)

applied as food packaging materials, these having antibacterial properties toward *E. coli* and *Staphylococcus aureus*.

Novel bionanocomposites based on chitosan, CMC, and ZnO nanoparticles (CH/CMC/ZnO bionanocomposites) were successfully prepared by casting method and used as packaging materials for soft white cheese. Soft white cheese was manufactured from buffalo milk, packaged within the prepared bionanocomposites films, and stored at 7 °C for 30 days. When using bionanocomposite films to package the cheese, compared with control treatment, no significant difference was observed in chemical properties of soft white cheese after the storage period. Also, CH/CMC/ZnO bionanocomposite packaging film has significant impact on pathogenic strains and presents large inhibition zones that ranged from 5 to 15 mm (Youssef et al. 2016). Jipa et al. (2012) induced antimicrobial properties to biodegradable bacterial cellulose (BC)-based films by incorporating sorbic acid (SA). The results indicated that the prepared films might be used as active materials for food packaging with controlled release of an antimicrobial agent.

14.2.6.3 Active Food Packaging Containing TPS

For reducing food spoilage multilayer materials are a practical choice because it can provide a much higher resistance to water and gas transfer than single-layered materials. TPS has excellent oxygen barrier properties as compared to other polymeric materials. A compatible mixture could offer impressive barrier properties by reducing food spoilage rates compared to conventional packaging materials. Fabra et al. (2016) obtained multilayer structures based on thermoplastic corn starch prepared by melt mixing containing bacterial cellulose nanowhiskers. The authors showed that the developed corn starch materials exhibit an important enhancement of oxygen barrier properties up 95%. Blending TPS with PLA is a good alternative to fulfil some requirements necessary for food packaging usage; TPS film has to be resistant to moisture content and to have good mechanical properties, while PLA film has to be flexible and to display excellent resistance to oxygen permeation. Nawab et al. (2017) investigate the effect of mango kernel starch coating on the quality and shelf-life of tomatoes. The quality assessment of the tomatoes was evaluated after every 4 days, and it was observed that using mango kernel starch as a packaging material is a natural and safe method to prolong the shelf-life of tomato fruit. Sanyang et al. (2016) prepared bilayer films from sugar palm starch and PLA without addition of any compatibilizer. The experimental results indicated that good mechanical and water barrier properties were obtained for the system with 50% starch and 50% PLA, which enhance their applicability for food packaging. Antimicrobial agents are used in TPS matrix to improve the quality of the foods by preventing the microbial growth. Campos-Requena et al. (2017) obtained by extrusion method nanocomposite films based on starch/montmorillonite/essential oil constituents (carvacrol and thymol), and it was evaluated to be potentially used as fresh strawberry packaging materials. The results indicated no considerable alteration of the quality parameters and organoleptic properties of strawberries with TPS/clay/carvacrol films as compared to control (strawberries without nanocomposite film), where evident growth of fungi is noticed after 5 days at room conditions (Fig. 14.4).

Active biodegradable films from cassava starch were synthetized by casting method (Medina-Jaramillo et al. 2017) and the potential of natural extracts such as green tea and basil to obtain active food packaging materials was evaluated. The authors concluded that incorporation of the natural extracts led to TPS films with low water vapor permeability retaining their flexibility and excellent thermal properties suggesting that starch films with tea or basil extracts can be used as coatings for any foods able to be cooked at temperatures <280 °C. Romani et al. (2017) evaluated the influence of oregano essential oil on rice starch/fish protein active packaging films

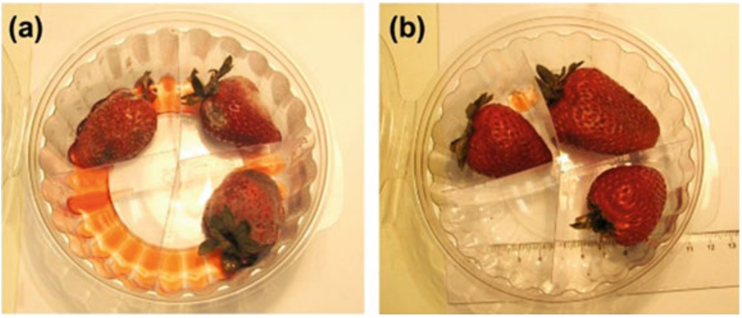


Fig. 14.4 In vivo antimicrobial assay in *Botrytis cinerea*-inoculated strawberries: (**a**) control without nanocomposite film, and (**b**) with TPS/MMT clay/carvacrol:tymol nanocomposite film (cavacrol:thymol 50:50) (Reproduced from Campos-Requena et al. 2017 with permission from Elsevier 2020)

obtained by solvent casting. The results showed that the ratio 50/50 of starch/protein was the most suitable to develop low cost food packaging materials having good mechanical properties (tensile strength 5.69 MPa, elongation 85.5%) and low water vapor permeability (0.18 g mm $\text{kPa}^{-1}\text{h}^{-1}\text{m}^{-2}$). The incorporation of oregano essential oil led to 63.1% inhibition of peroxidase enzyme (responsible for enzymatic browning in foods), suggesting that the starch/fish protein blends incorporated with oregano essential oil are suitable to be used as anti-browning packaging films, increasing the shelf-life of fruits and vegetables such as potatoes, apples, or pears.

Another suitable antimicrobial agent for starch packaging materials is chitosan. Mendes et al. (2016) evaluated the chitosan effect on corn starch–chitosan blends obtained by extrusion method. The experimental results indicated that it was possible to produce corn starch–chitosan blends by extrusion and the addition of chitosan determined increased thermal stability of films. Moreover, chitosan acted as a plasticizer for thermoplastic starch matrix, increasing the elongation at break, and decreasing tensile strength and elastic modulus. A package prototype for perishable food products such as strawberries, ricotta, and flavored breads from active films based on thermoplastic corn starch and chitosan oligomers was successfully developed by spraying method (Castillo et al. 2017). Various examples of AP materials based on bio-derived biodegradable plastics are listed in Table 14.2.

14.3 Bio-Derived Non-biodegradable Plastics

Non-biodegradable bio-based plastics is a class of biopolymers that comprises plastics like bio-based poly(ethylene) (bio-PE), bio-based poly(ethylene terephthalate) (bio-PET), bio-based polyamides (bio-PAs), poly(trimethylene terephthalate) (PTT), bio-based poly(propylene) (bio-PP), and, more recently, poly(ethylene furanoate) (PEF). They are fully or partially obtained from bio-based building blocks and present similar chemical structure and properties as petrochemical counterparts. “Green polymers” represent around 57% of the total bio-plastics in world production. Among them, bio-PET is the most produced bio-plastic, accounting for 26% of the total production (Monteiro et al. 2009; Taha and Ziegmann 2006; Montava-Jordà et al. 2019). Most common applications of bio-based plastics are in food packaging and service ware, bags, fibers/nonwovens, and agriculture, the target application being for short functional life products (packaging and disposables). In order to minimize the environmental impact of food packaging products significant efforts are oriented towards the replacement of traditional packaging with new lower carbon footprint materials derived from renewable resources. Currently, from this class of plastics bio-PE and bio-PET (bio-based content 30%) are the main materials used in packaging. An important application for bio-PE is in obtaining flexible films, while bio-PET is used as rigid material (e.g. bottles) (van den Oever et al. 2017).

Table 14.2 Recent examples of active packaging materials based on bio-derived biodegradable plastics

Bio-plastic	Active component	Obtaining procedure	Type of tested food/functionality	Target function of the active packaging	References
Poly(lactic acid) or poly(hydroxyalkanoates) based materials					
PLA	ZnO nanoparticles, *Zataria multiflora Boiss* and *Menta piperita L.* essential oils	Solvent casting	*O. ruber* fish fillets	Antibacterial and antioxidant, shelf-life extension	Heydari-Majd et al. (2019)
PLA and PBAT blends	Trans-cinnamaldehyde	Cast extrusion	Bread	Antibacterial and antifungal	Srisa and Harnkarnsujaritm (2020)
PLA	Thymol, kesum, and curry	Solvent casting	Chicken meat	Antimicrobial, enhanced shelf-life	Mohamad et al. (2020)
PLA and poly(ethylene oxide)	Biomass of the microalga Spirulina sp. LEB 18	Electrospinning	Not defined	Colorimetric pH indicator, food quality evaluation	Kuntzler et al. (2020)
PLA	Sodium chlorite and glucono delta-lactone	Solvent casting	Tomato	Antimicrobial	Zhou et al. (2020)
PLA and PHB	Angelica essential oil	Solvent casting	Peach	Antioxidant and enhanced barrier properties	Jiang et al. (2020)
PLA	Selenium	Solution casting	Food pathogens	Antioxidant and antimicrobial activity	Lu et al. (2020)
PLA and ethylene–vinyl acetate–glycidyl methacrylate	Star-like lignin microparticles	Surface grafting and melt blending	Pathogenic microorganisms and barrier properties	Antioxidant	Yang et al. (2020)
PLA and PHB	Fennel oil	Melt blending and casting	Oysters	Antimicrobial	Miao et al. (2019)

(continued)

Table 14.2 (continued)

Bio-plastic	Active component	Obtaining procedure	Type of tested food/functionality	Target function of the active packaging	References
PLA and poly(butylene succinate)	Oregano essential oil	Melt blending in a twin extruder	Lettuce	Preservation of phytosterol and shelf-life extension	Llana-Ruíz-Cabello et al. (2019)
Materials containing cellulose or cellulose derivatives					
Chitosan/cellulose acetate/phthalate	ZnO nanoparticles	Casting method	Photogenic microorganisms	Barrier and antimicrobial properties	Indumathi et al. (2019)
Chitosan/CMC	ZnO nanoparticles	Casting method	Soft white cheese	Antimicrobial properties	Youssef et al. (2016)
CMC/sodium alginate	Ag nanoparticles	Casting method	*K. pneumoniae* and *S. pyogenes* pathogens	Antimicrobial	Su et al. (2017)
CMC	Thai rice grass extract	Microencapsulation	Green tea leaves	Extended food shelf-life; antioxidant	Rodsamran and Sothornvit (2018)
CMC	Olive oil	Drop-wise	–	Improved moisture barrier and water vapor permeability	Rodsamran and Sothornvit (2017)
BC/PVA	*Aspergillus niger*	BC gel dispersed in PVA solution	–	Improved mechanical strength and better biodegradability	Stoica-Guzun et al. (2011)
BC/sorbic acid	Sorbic acid	Casting method	–	Controlled released of antimicrobial agent	Jipa et al. (2012)
Thermoplastic starch-based materials					
TPS	Oregano essential oil Fish protein	Solvent casting	Mechanical, barrier, antioxidant testing	Anti-browning	Romani et al. (2017)

TPS	Chitosan oligomer	Coating by spraying	Strawberries, ricotta, and flavored breads	Antimicrobial, diffusion testing	Castillo et al. (2017)
Thermoplastic cassava starch/linear-low-density poly(ethylene)	Sappan and cinnamon herbal extracts	Blown-film extrusion	Beef meat	Antimicrobial	Khumkomgool et al. (2020)
PHB-TPS	Eugenol	Melt mixing	Antioxidant test Antifungal test against *Botritys cinerea*	Antioxidant and antifungal	Garrido-Miranda et al. (2018)
Acetylated cassava starch/linear low-density polyethylene	Sodium nitrite	Blown-film extrusion	Pork meat	Antimicrobial, antioxidant	Chatkitanan and Harnkarnsujarit (2020)

14.3.1 Bio-Based Poly(ethylene)

Bio-PE is a polyolefin obtained from an environmentally friendly product, bio-ethylene, which is processed in several steps from renewable raw material, like sugar cane, used to produce ethanol. Bio-PE is produced by bio-ethylene polymerization and has the same properties and applications as for conventional PE and can be differentiated from plastics by using the radiocarbon method (Greene 2014; Krauter et al. 2019). From a technical point of view, bio-PE has the same properties and characteristics as conventional petrochemical-based PE and can be processed using the equipment designed for PE, without the need for an additional investment into equipment. Also, a very important advantage for the manufacturing industry is represented by the fact that bio-PE can be recycled in the same recycling stream that is already in place for PE (Mendieta et al. 2020). Bio-based materials are mostly hydrophilic, and the humidity conditions had an influence on the gas barrier properties (when the humidity increases, the gas permeability of bio-based materials may also increase) (Pandit et al. 2018). For non-biodegradable bioplastics (bio-PE, bio-PET, and bio-based polyamide (bio-PA)), because of the same chemical structures as petroleum-based plastics (PE, PET, PA), the gas permeability (water vapour, O_2, and CO_2) is also the same. In case of bio-PE and bio-PET, in order to improve the O_2 and moisture barrier properties, lamination technique was combined with biaxial orientation. Due to their high degree of crystallinity and ordered packaging structure, biaxially oriented PE or PET (BOPE or BOPET) plays an important role in obstructing gas permeability. To increase barrier properties, the biaxially oriented bio-PE and bio-PET films have been further metalized (Chang and Brandmeier 2011; Sakellarides and Chang 2013; Jariyasakoolroj et al. 2019).

14.3.2 Bio-Poly(Ethylene Terephthalate)

PET is one of the most commonly used polymers and is made from ethylene glycol (EG) and terephthalic acid (TPA) molecules. One of the possible routes to obtain bio-EG is by synthesis from hydrolysis of ethylene oxide, produced by the oxidization of bio-ethylene, resulted in turn through glucose fermentation, and followed by dehydration (Rebsdat and Mayer 2001). Thus, a partially bio-based polymer is obtained, and the percentage of the bio-based monomers varies depending on the stoichiometry of the reaction. Another method to synthetize bio-EG is by using different types of microorganisms, e.g. bacteria, in high yields using xylose as a substrate (Salvador et al. 2019). Although EG can be obtained from renewable raw materials (bio-ethanol), there is no cost-effective way to produce bio-based terephthalic acid, thus making bio-poly(ethylene terephthalate) (bio-PET) only 30% bio-based. Due to its properties, bio-PET is used like conventional PET in various food and beverage packaging applications (Greene 2014; Krauter et al. 2019).

Due to its physical and mechanical properties bio-PET is suitable for fibers (65%) and packaging (35%) applications (e.g. bottles (76%), films (13%), and containers

(11%)). Partial bio-PET occupies an important part of the bio-plastics rigid packing materials (approximately 58%), but due to a slower degradability, it entails serious environmental issues, when it is used for short time applications, like food packaging (Xiao et al. 2015; Nakajima et al. 2017; Siracusa and Blanco 2020). Although it is non-degradable in nature, it can be recycled (Roohi et al. 2018).

Recently, efforts have been made to replace petro-based terephtalic acid with bio-based TPA in order to obtain a 100% bio-PET. Depending on the biomass sources used in bio-PTA production, the amount of greenhouse gas emissions during the life cycle of bio-PET 100 can be between 25 and 58% lower than that from petroleum-based PET (Hwang et al. 2020).

14.3.3 Bio-Polyamides

Polyamides represent an important class of polymers with multiple applications. In the context of developing sustainable polymers from renewable resources, many polyamides based on natural building blocks have been described. These bio-based starting materials can provide sustainability and special structural features to the resulting polymers and their properties, e.g. side groups, functionalities, or stereo-information. Bio-based polyamides (bio-PAs) are a new class of bioplastics that are derived from renewable resources such as natural fats and oils. The majority of these bio-amides are based on sebacic acid with the exception of polyamide 11 (also called Nylon 11 or Polyundecanolactam) which is based on undecylenic acid. In the production of commercially available bio-PA, castor oil from *Ricinus communis* plant is used as a renewable feedstock (Mutlu and Meier 2010; Winnacker and Rieger 2016). Castor beans have high oil content (40–60%) that consists mainly of the C18 fatty acid ricinoleic acid (85–90%), which is available in its triglyceride ester. The double bond and hydroxy group of ricinoleic acid provide different possibilities for chemical modifications (Ogunniyi 2006). Modifications can lead to building blocks such as sebacic acid, aminoundecane acid, or decamethylenediamine (Kuciel et al. 2012; Kyulavska et al. 2017).

Bio-polyamides can be synthesized by different processes like (a) polycondensation of diamines and dicarboxylic acids with elimination of water, (b) polycondensation of amino carboxylic acids as bifunctional monomers (e.g. PA 11), (c) from bio-based acid and petroleum-based amine (e.g. PA 410, PA 610) or from both components obtained from biomass (e.g. PA 510, PA 1010), and (d) ring-opening polymerization of lactams (e.g. PA 4, PA 6, PA 66) (Thielen 2010).

Due to its great properties like excellent chemical resistance and electrical properties, good abrasion resistance, good processability, high melting point, high stiffness and strength in dry conditions, high heat distortion temperature, and toughness, bio-PA 66 is one of the most extensively used PAs. Its major disadvantage is the relatively high moisture absorption, which makes the dimensional stability, tensile strength, and stiffness in humid environment to be diminished (Ahmadi et al. 2010; Kyulavska et al. 2017). Bio-PA66 has good thermal and mechanical properties, with a melting point of 265 °C, being the second aliphatic polyamide having the highest melting point. According to ISO 1110, moisture absorption is relatively high (8–9 wt%), but lower than that for PA6 (10 wt%) and PA46 (13 wt%) (International Organization for Standardization 1995). The commercial success of Bio-PA 11 and Bio-PA 12, despite the fact that the price is significantly higher than PA 6 and PA 66, is due to their properties like excellent chemical resistance, superior durability and aging, flexibility, good impact, and thermal resistance (Ensinger 2012; Pandit et al. 2018).

14.3.4 Poly(Trimethylene Terephthalate)

Poly(trimethylene terephthalate), another bio-based polymer, is a linear thermoplastic aromatic polyester obtained through condensation of terephthalic acid or its dimethyl ester with 1,3-propanediol obtained from plant waste through a biotechnological method, resulting a partially bio-derived PTT. Bio-PTT has mechanical properties similar to PET and poly(butylene terephthalate) (PBT) but is biodegradable like aliphatic polyesters (Samperia et al. 2004). The spinning processability of PTT determined its extensive application in obtaining products like fibers, carpets, and textiles and for sanitary and medical applications. PTT can also be used as construction materials or as packaging material films (Pisula et al. 2006; Rydz et al. 2018). PTT has excellent physical properties, chemical resistance, low moisture absorption, dimensional stability, processability, and recyclability. Based on these properties' comparison with some of the conventional polymers, it can be concluded that PTT market has a high substitution potential for PA (nylon) and PBT while moderate in case of PC, PP, and PET. Based on biodegradability, bio-PTT can also be used as alternative for PLA, PHA, CA, and cellophane films in certain applications. Substitute potential for bio-based over petroleum-derived PTT can be 100% (Kobayashi et al. 2011).

14.3.5 Bio-Derived Non-degradable Plastic Materials in Active Food Packaging

The research performed in active food packaging applications that involve bio-derived non-degradable plastics is scarcely developed. One possible reason is the fact that the main focus is on finding and development of renewable resources for the bio-constituent building blocks and new economical feasible synthesis methods. Hence, few examples of active food packaging including bio-derived non-degradable plastics are reported until now—Table 14.3. In general, the bio-derived non--degradable plastics were used to obtain conventional packaging, not active packaging.

South America's largest chemicals and plastics supplier, Braskem, a major producer of **bio-based PE**, produces a wide range of bio-based high-density poly (ethylene) (bio-HDPE) and linear low-density poly(ethylene) (LLDPE) resins, which can be used in applications like food industry, personal care, cleaning,

Table 14.3 Examples of active food-packaging materials based on bio-based non-biodegradable plastics

Bio-plastic	Type of active component	Obtaining procedure	Type of function tested	References
Bio-PE/ chitosan	Chitosan and ZnO nanoparticles	Casting method	Antibacterial properties pathogens bacteria *E. coli* and *S. aureus*	Al-Naamani et al. (2016)
Bio-PE/ chitosan	Chitosan and TiO_2 nanoparticles	Casting method	Better barrier properties and tensile strength	Kaewklin et al. (2018)
HDPE/ cellulose	Lignin	Injection molding	Oxidation induction time extension	Guilhen et al. (2017)
Biaxially oriented PE or PET	Lamination by resin or metal layer	Embadiment	High gas and moisture barrier	Chang and Brandmeier (2011)

cosmetics, and automotive industries. Currently, Braskem has several partners including Coca-Cola, Danone, Procter & Gamble, TetraPack, Johnson & Johnson, Acinplas, Shiseido, Toyota Tsusho, PetroPack, and NobelPack, among others (Byun and Kim 2014) that use bio-derived PE for their products. Since 2011, probiotic yogurt brand Actimel (Danone) (Fig. 14.5a) uses only bottles made from 95% or more bio-based PE and 5% or less non-bio-based plastic. Although bottles are not biodegradable, they are 100% recyclable. The juice maker Odwalla (Odwalla PlantBottle Coca-Cola) (Fig. 14.5b) has also begun using juice bottles that comprise 96% or more bio-based PE and 4% or less non-bio-based plastic (Byun and Kim 2014).

Bio-PET is already produced using resin with further formation into a bottle shape at the industrial scale. In general, biodegradable bottles are not recyclable and are an issue with current recycling systems. In 2009, Coca-Cola introduced PlantBottle, which is manufactured of materials that are up to 30% plant based—Fig. 14.6. It has the same chemical structure and functionality as traditional PET plastics, but the biggest advantage is that it does not sacrifice the performance or recyclability of traditional PET. Currently, PlantBottle packaging is available in nine countries including Denmark, the United States, Canada, Japan, Brazil, Mexico, Norway, Sweden, and Chile. In Canada, PlantBottles are used to package Dasani, Coca-Cola, Diet Coca-Cola, Coca-Cola Zero, Sprite, Fresca, and Barq's. The use of PlantBottle packaging provides a reduction of CO_2 production up to 19% (Byun and Kim 2014; Jariyasakoolroj et al. 2019; Niaounakis 2015). In 2011, Heinz licensed the PlantBottle technology from Coca-Cola for their ketchup bottles in the United States and Canada. In 2012, a strategic working group focused on accelerating the development and use of 100% plant-based PET materials in their products was formed by Coca-Cola, Ford Motor, Heinz, Nike, and Proctor & Gamble and was named Plant PET Technology Collaborative (PTC) (Byun and Kim 2014). In 2011,

Fig. 14.5 (**a**) Actimel's new bottle made from Braskem's bio-based PE (Fadigas 2011) and (**b**) Odwalla's juice PlantBottle (Mohan 2011, © 2020 PMMI Media Group)

Fig. 14.6 Bio-PET (30%) in packaging applications (Gotro 2012, © 2020 Innocentrix, LLC All)

PepsiCo successfully produced the world's first **100% Bio-PET** made from switchgrass, pine bark, and corn husks—Fig. 14.7. Bio-PET packaging has the same structure, appearance, and properties as traditional petroleum-based PET products. They are also working on producing bio-based PET from other natural resources such as orange peels, oat hulls, and potato scraps (Jariyasakoolroj et al. 2019; Byun and Kim 2014).

Although **PA** (known in everyday life as nylon) is valued for its tear resistance, elasticity, and good barrier properties, it is not commonly used in food contact material, its applications in food packaging being limited (Greene 2014; Krauter et al. 2019), PA11 and PA12 being the only ones used in food packaging applications (Kyulavska et al. 2017). DuPont has commercialized **bio-PTT** for applications in packaging and high performance engineering and has expanded its renewably sourced packaging including DuPont™ Biomax® TPS thermoplastic starch and DuPont™ Biomax® PTT injection-moldable resin. Biomax PTT contains as much as 35% renewably sourced content for packaging applications and is suitable for injection-molded containers, cosmetic packaging, and other parts where polyesters are used (DuPont Packaging 2008).

Fig. 14.7 100% Bio-PET Pepsi Bottle (Plastics Today Staff 2011, © 2020 Informa Markets)

14.4 Conclusion

This chapter provides an overview on recent advancements in the bio-derived bioplastics used in active food packaging. The bioplastics serve as a green replacement for the use of traditional plastic-based packaging materials. The two discussed groups of bio-based bioplastics, namely, biodegradable and non-biodegradable, may represent a solution to overcome the drawbacks associated with the conventional plastics used in food packaging (environmental issues, health problems, etc.). Recently, the bio-derived bioplastics in active food packaging gained an important attention, being used as support matrices or carriers for bioactive compounds, ensuring appropriate mechanical and barrier properties, and some of them can impart bioactive functions.

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Synthetic Bioplastics in Active Food Packaging

15

Elena Butnaru, Elena Stoleru, and Anamaria Irimia

Abstract

With the increasing demand for plastics and rising pressure for their safe disposal, biodegradable plastics gained a lot of attention in the recent years. The synthetic biodegradable plastics class comprises polymers that have in their structure specific chemical bonds (e.g. ester, amide, and urethane) that can be cleaved by microorganisms. Over the last 10 years, the trend line in the food industry indicates a continuing demand for healthy foods and more natural alternatives to prolong the shelf life of foods. Hence, the present chapter reviews the performance of synthetic biodegradable plastics in food packaging, with a certain attention on the blends and composites of poly(vinyl alcohol), poly(caprolactone), and other polymers in active food packaging. Moreover, the environmental impact (e.g. end-of-life options, biodegradation) of the synthetic bioplastic-based food packages is also discussed. Although at this moment these biodegradable synthetic plastics are still far from being an economical and feasible alternative to compete with conventional plastics in the near future there is a positive outlook if the research progress in this field is taken into account.

Keywords

Synthetic polymers · Bioplastics · Food packaging · Biodegradable · Applications

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15.1 Introduction

For several decades now the plastics govern various production sectors, from commodity items to specialty materials. Plastics have many excellent properties, such as mechanical properties, as well as chemical, weathering, and biodegradable resistance, that make them suitable for extensive application in various fields. However, the same properties which made them exemplary for application in numerous domains, e.g. durability in the case of construction materials or commodities, may cause long-term problems (mainly negative impact on the environment) (Potts et al. 1973). In general, food packages are made from fossil-based plastics that are scarcely or difficult to recycle and give rise to waste disposal issues, consequently imposing environmental burden (Jem and Tan 2020). In these circumstances, the use of biodegradable plastics in food packaging could represent a viable alternative/solution to waste accumulation. The class of biodegradable plastics can be grouped in two categories, considering the source of feedstocks, namely, synthetic (or petroleum derived) and natural (or biological derived) polymers (Siracusa et al. 2008). The synthetic biodegradable plastics class comprises polymers that have in their structure specific chemical bonds (e.g. ester, amide, and urethane) that can be cleaved by microorganisms. In general, the polymers with hydrolysable backbones are prone to biodegradation in specific environments (Vroman and Tighzert 2009).

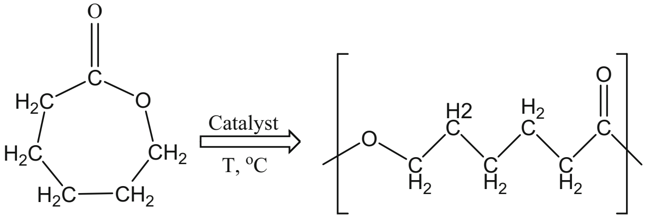
In recent years, the role of packaging has changed or become more complex. It has started from a passive role, assuring the transport and preventing physical deterioration and aiming nowadays an active role, such as refined protective function to prolong the shelf life of the food (Yildirim et al. 2018). Active packaging (AP) involves the use of polymer materials that act as a support for different active compounds (e.g. natural extracts) that can be incorporated during the manufacturing process of the packaging (Bassani et al. 2019; Kuorwel et al. 2015). This chapter is focused on bioplastics used in AP that are typically obtained from petrochemical feedstocks (conventional synthesis from synthetic monomers) and possess weak linkages that are vulnerable to enzymatic attack leading to biodegradation of the polymer (Coles et al. 2011). This group of bioplastics includes various polymers, such as poly(caprolactone) (PCL), poly(vinyl alcohol) (PVA), poly(butylene adipate-co-terephthalate) (PBAT), etc.

15.2 Types of Fossil-Based Biodegradable Polymers

15.2.1 Poly(Caprolactone)

15.2.1.1 Properties and Limitations

Poly(caprolactone) is an aliphatic polyester, which became popular in the last 10 years, being an eco-friendly material which proved that can be a viable alternative to traditional plastics. It is a thermoplastic polymer, usually synthesised by ring-opening polymerization of ε-caprolactone in the presence of anionic or cationic



Scheme 15.1 The structure of caprolactone and PCL

initiators and coordinating or enzymatic catalysts (Żółtowska et al. 2015) at temperatures higher than 120 °C (Scheme 15.1).

PCL is semi-crystalline and hydrophobic, with melting temperature ranging between 59 and 64 °C and a glass-transition temperature of 60 °C. PCL is soluble in different solvents at room temperature like toluene, chloroform, etc. (Zhang et al. 2019). In Europe, PCL is mainly produced by Solvay Interox LTD. under the trade name of "CAPA". PCL's properties are strongly related to its molecular weight. CAPA PCL is a high molecular weight polymer with mechanical properties (elongation at break >1100% and tensile strength of 20–40 MPa) similar to LDPE (low-density poly(ethylene)) (elongation at break 500–725% and tensile strength 9.7–17.2 MPa) (Salah Fawzi 2019). PCL has been approved by the United States Food and Drug Administration (FDA) as food contact material (Cheeseman 2017) and is particularly used in food packaging industry for its biodegradability, blend compatibility, good rheological and viscoelastic properties (Matzinos et al. 2002). The mechanical properties of PCL are not satisfying (because of chain flexibility), and the barrier properties to water and gases are limited (Khan et al. 2013); thus it restricts its wide use in the packaging field. Hence, many researchers studied to overcome these drawbacks by blending PCL to other polymers such as starch (Khalid et al. 2018), PLA (poly(lactic acid)) (Plackett et al. 2006), chitosan (Wang et al. 2019), and others, to produce materials with good mechanical and gas barrier properties in order to minimize deterioration, prolonging the shelf life by increasing the quality and safety of food products.

PCL is easy to be manipulated into various forms including blown films, fibers, microspheres, nanocapsules, and nanoparticles (Woodruff and Hutmacher 2010; Zambrano-Zaragoza et al. 2011). At laboratory scale, most of the packaging materials are obtained by solution casting technique, but the extrusion method is normally used in the industry, because it is a non-solvent method and has shorter processing time (no solubilization, casting, and drying steps). However, these conventional methods (solution casting, melt processing) have some limitations regarding the incorporation of volatile active compounds into polymeric matrices; using the solvent casting method can result in the evaporation of the active substance with the solvent, and melt processing implies high temperatures which can result in degradation reactions. These limitations can be avoided by using supercritical solvent impregnation (Milovanovic et al. 2018).

15.2.2 Poly(Vinyl Alcohol)

15.2.2.1 Properties and Limitations

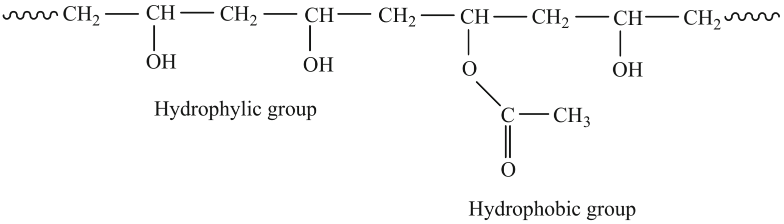
Poly(vinyl alcohol) has a melting point of 180–190 °C, a molecular weight of 20–200 Da, and a degree of hydrolysis of 80–99%. The total number of repeating monomer units in PVA (Scheme 15.2) ranges from 500 to 5000 (Li and Yang 2018). It is usually obtained by the polymerization of vinyl ester monomer because it cannot be prepared from vinyl alcohol, being unstable and easily tautomerized into acetaldehyde. PVA could be also be prepared from either the complete or partial hydrolysis of poly(vinyl acetate) where the acetate groups are removed, resulting in a PVA highly soluble in water and insoluble in organic solvents. Incomplete removal of the acetate groups results in a less water soluble polymer and more soluble in organic solvents (Hassan et al. 2002). Fully hydrolyzed PVA (98–99.8%) can dissolve in water only at temperatures higher than 80 °C. The solubility of partially hydrolyzed PVA (85–90%) is dependent upon its molecular weight (Lyoo et al. 2003).

The physical properties of PVA are strongly related to the degree of hydrolysis, crystallization, molecular mass, or moisture. An increase in the molecular weight and degree of hydrolysis improves the viscosity, film forming ability, tensile strength, and resistance to solvents (Aslam et al. 2018).

15.2.2.2 Processability and Compatibility

PVA can be processed by solvent casting and electrospinning methods to make films and fibers for food packaging. Solvent casting is the oldest method used for obtaining uniform packaging films. Also, polymer blending is an effective method to have new materials with modified physical and mechanical properties compared with films made of individual components. It was intensively proved the compatibility of PVA with natural polymers (e.g. chitosan, cellulose, starch, etc.) (Muppalla et al. 2014; Părpăriță et al. 2014; Nwabor et al. 2020), inorganic particles (e.g. clay, silver nanoparticles) (Butnaru et al. 2016), antioxidants, and antimicrobial agents (e.g. basil leaves extract, grapeseed extract, thyme essential oil) (Singh et al. 2018) for obtaining novel materials with improved performances for active food packaging.

Singh et al. (2018) prepared PVA bio-nanocomposite films incorporated with cellulose nanocrystals and basil leaves extract, by solvent casting method. The obtained films showed antimicrobial and antioxidant activity attributed to basil



Scheme 15.2 Chemical structure of PVA

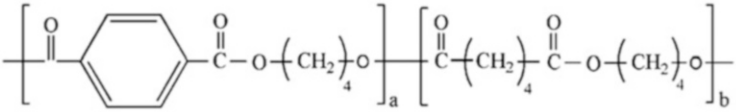
leaves extract. Moreover, the mechanical properties of pure PVA films were relatively low, being not suitable for packaging applications. The incorporation of cellulose nanocrystals and basil leaves extract into the PVA matrix resulted in superior tensile strength and water vapor permeability values, proving that the obtained films are suitable for active food packaging applications.

15.2.3 Poly(Butylene Adipate-Co-Terephthalate)

15.2.3.1 Properties and Limitations

Poly(Butylene Adipate-Co-Terephthalate) is another petroleum-derived polyester, 100% biodegradable, obtained by polycondensation reaction of 1,4-butanediol, adipic, and terephthalic acids, requiring long reaction time, high vacuum, and high temperature (>190 °C)—Scheme 15.3. PBAT used in packaging applications is commercially available as Ecoflex produced by BASF, Germany, being the most commercial utilized aliphatic-aromatic polyester (Díaz et al. 2014). Ecoflex is designed to obtain films similar to LDPE, ideal for trash bags, food containers, and film wrapping, having short decomposition time within a few weeks in compost or soil.

Compared with other polyesters, such as PLA and poly(butylene-co-succinate), PBAT has higher flexibility, with values for Young modulus of 20–35 MPa, tensile strength of 32–36 MPa, and elongation at break of approximately 700%, being similar to those of LDPE. These properties make PBAT suitable for food packaging applications (Ferreira et al. 2019), including cling wrap roll. Therefore, PBAT seems to be a suitable choice for replacing LDPE films due to perfect balance between the thermo-mechanical properties and biodegradability. The main limitations of this polymer are its poor thermal and mechanical resistance, its high water vapor permeability, and its price, being three times more expensive than LDPE. For these problems, several approaches have been addressed, including dispersion of nanofillers into polymer matrix, being found to be a successful method for lowering water vapor permeability. The results obtained by Li et al. (2018) showed that organically modified montmorillonite significantly lowered water vapor permeability values when added to PBAT nanocomposites up to 13 wt% and could be a proper alternative for poly(ethylene) (PE) films in packaging. Also, Mondal et al. (2014) successfully obtained PBAT/nanocomposite films and registered a 25% decrease in water vapor permeability of PBAT with the addition of 4 wt% organically modified nanoclay.



Scheme 15.3 Chemical structure of PBAT

15.2.3.2 Processability and Compatibility

PBAT possesses strong processing stability and can be used as it is or blended with other polymeric materials through traditional manufacturing methods such as solvent casting, extrusion, or blowing film. The melt flow index at 190 °C/2.16 kg is about 4 g/10 min, making PBAT useful for blowing film applications (Pivsa-Art et al. 2011). Due to its high flexibility, PBAT is used as an additive for more rigid biodegradable plastics, such as PLA, PBS, and PP in order to increase the toughness, rigidity, and its biodegradability. Pukpantas and Sirisinha (2012) prepared PLA/PBAT blends by melt-blending using a twin-screw extruder; the Irganox antioxidant was incorporated in the PLA/PBAT blends, and it was observed a significant improvement in elongation at break for the 50/50 blend containing 0.3% antioxidant.

15.3 End of Life Options of Synthetic Bioplastics

15.3.1 Biodegradation of PCL-Based Materials

Due to its semi-crystalline nature and the presence of five hydrophobic –CH_2 moieties in its repeating units, PCL is mainly biodegraded by hydrolysis reactions and therefore, exhibits a longer time (2–4 years) for its complete degradation (Woodruff and Hutmacher 2010). Its slow degradation rate and water resistance make PCL acceptable as a packaging material. On the other hand, enzymatic degradation occurs at the surface of the polymer with lipases and esterases, effectively accelerating the degradation of PCL (Murray et al. 2015). Thus, in the presence of *Pseudomonas lipase* has been reported the complete degradation of PCL in only 4 days (Gan et al. 1999). The degradation of PCL films was investigated using a thermotolerant *Aspergillus sp.* strain ST-01, and it was found that the PCL films were degraded after 6 days at 50 °C; succinic, butyric, valeric, and caproic acids were find to be the main degradation products (Sanchez et al. 2000). In natural environments, such as compost, sea water, and pond, it was found that the degradation of PCL is the primarily result of enzymatic hydrolysis, which plays a predominant role in the degradation process (Krasowska et al. 2016). Moreover, it was found that the biodegradability in soil of PCL-based composites was accelerated by the incorporation of grapefruit seed extract and attributed to a lower crystallinity of the composites (Lyu et al. 2019).

15.3.2 Biodegradation of PVA-Based Materials

PVA is biodegradable under both aerobic and anaerobic conditions. It was found that 55 microorganisms are suitable to degrade PVA (Leja and Lewandowicz 2010). *Pseudomonas* species were the first microorganisms identified to degrade PVA. The biodegradation mechanism of PVA consists in the oxidation of the 1,3 hydroxyl groups mediated by oxidase and dehydrogenase type enzymes. Firstly, monoketone or diketone structures are formed, followed by the carbon-carbon bond cleavage promoted by specific β-diketone hydrolase, resulting carboxyl and methyl ketone end groups (Falkiewicz-Dulik et al. 2015). Aerobic microbial degradation of PVA/starch films proposed for food packaging applications was studied by Jecu et al. (2010). It was found that *Aspergillus niger* was the most able to grow and to degrade the PVA-based materials among the 11 microorganisms tested.

The study, performed by Cano et al. (2016), highlighted the effect of incorporating antimicrobial substances (e.g. neem oil, oregano essential oil, and silver nanoparticles) into PVA-starch-based materials on the biodegradation behavior. The authors observed that the biodegradation capacity of starch-PVA samples was not affected by the neem and oregano essential oil content, while the biodegradation of the samples containing silver species was seriously retarded.

15.3.3 Biodegradation of PBAT-Based Materials

Many researchers have reported on biodegradation of aliphatic-aromatic polyesters (Nakajima-Kambe et al. 2009; Kijchavengkul et al. 2010). The biodegradation process occurs via enzymatic action of microorganisms such as bacteria, fungi, and algae or by chemical hydrolysis/thermal degradation. Biodegradation rate in the PBAT depends on the terephthalate content in the polymer. Witt et al. (1997) reported that the biodegradation rate decreases with the increasing concentration of terephthalic acid in PBAT (more than 55 mol%). Kijchavengkul et al. (2010) found that the aliphatic butylene adipate (BA) unit was more susceptible to hydrolysis and biodegradation than that of the aromatic butylene terephthalate (BT) unit; also, the biodegradation rate of PBAT was higher in manure compost than in yard or food waste compost. Wu (2012) found that the biodegradation rate of PBAT composite is faster than that of the neat PBAT. The biodegradability of PBAT in the presence of active agents was studied by Freitas et al. (2017); the authors assessed from evolved CO_2 the influence of montmorillonite clay on the biodegradation of PLA/PBAT blends in soil. The test results indicated that after 126 days of soil burial, the clay delayed the blends biodegradation and only some cracks on their surfaces were observed. A biodegradation study of PLA/PBAT blends in the form of films and disposable bags was conducted under industrial composting conditions by Musioł et al. (2018). Also, an abiotic degradation laboratory test was carried out in distilled water at 70 °C in order to show that it is important to control both moisture and temperature. The results indicated that the degradation process started after 21 days of incubation under industrial composting conditions.

15.4 Active Food Packaging Applications of Synthetic Biodegradable Plastics

Fossil-based biodegradable polymers have seen limited use as AP materials because are too expensive to be a fine replacement of common plastic materials (e.g. polyethylene terephthalate (PET), high-density polyethylene, etc.). Therefore, they are usually blended with other polymers (e.g. starch, cellulose, poly(lactic acid), etc.) (Zhong et al. 2020) to improve their properties and to obtain a strong and successful packaging material while reducing material cost. PCL is usually blended with biopolymers (e.g. starch) and is used as a food contact material and PVA is used as a coating material, while PBAT is used as fast food disposable packaging films (Halonen et al. 2020).

15.4.1 Poly(Caprolactone)-Based Food Packaging Materials

PCL is used for coatings or encapsulation of active agents due to its biocompatibility, permeability, and biodegradability (Choi et al. 2009). Fish oil-loaded PCL nanocapsules were prepared using an emulsion–diffusion method by Choi et al. (2010). The authors concluded that PCL efficiently retarded the release of fish oil in liquid or powder form and had good ability to protect the fish oil because of its water insolubility. The potential for extending the shelf life of food products was studied by Dumitriu et al. (2017), and the antioxidant effect of electrospun fibers obtained from PCL and vitamin E was proved. Lyu et al. (2019) successfully obtained PCL composite packaging films incorporated with grapefruit seed extract (GSE) as an antimicrobial agent, using a twin-screw extruder. The antibacterial effect of the PCL/GSE films on cheddar cheese inoculated with *Listeria monocytogenes* during storage at 4 and 25 °C was also studied. The results were compared with polypropylene/polyethylene based commercial cheese packaging films. It was found that PCL/GSE 5% film caused reduction in bacterial cell number in the cheese samples after 5 days.

PCL composite films with sodium metabisulfite as active compound were also prepared by melt compounding and melt-extruding using a twin-screw extruder (Jeong et al. 2020). The results demonstrated that the use of PCL/sodium metasulfite films significantly reduced the browning reactions of freshly cut apple and possesses excellent oxygen scavenging, antioxidant, antimicrobial, and antifungal capacity, being considered suitable for active food packaging applications. Sogut and Seydim (2018) prepared an active bilayer film obtained from chitosan and PCL containing nanocellulose and grape seed extract, as a promising material to transfer functional compounds when used as an active packaging material. Also, chitosan- and PCL-based bilayer films provided an extended shelf life of at least 3–6 days. An interesting study was conducted by Salević et al. (2019) where novel materials based on PCL containing a solid dispersion of sage extract were developed by annealing of the ultrathin electrospun fibers. The authors showed that the proposed systems have great potential in active food packaging applications with the role of preventing oxidation processes and microbial growth. Biodegradable nanocomposite films loaded with zinc oxide and clove essential oil on the polylactide/poly(ethylene glycol)/poly(caprolactone) (PLA/PEG/PCL) matrix using the solution cast technique were developed by Ahmed et al. (2019). The efficacy of the nanocomposite films was tested against *Staphylococcus aureus* and *Escherichia coli* inoculated in scrambled egg, and results indicated that the polymeric film exhibited the highest antibacterial activity during 21 days storage at 4 °C. It was also observed that the introduction of PCL into PLA/PEG significantly improved the mechanical strength of the blend.

Modified atmosphere packaging (MAP) is a technology used to extend the shelf life of perishable foods such as fruits or vegetables (Yun et al. 2017). In a recent study, an antimicrobial film based on PCL, cinnamaldehyde, and pomegranate methanolic extract, in a modified atmosphere (100% N_2), was successfully obtained with the purpose to extend refrigerated storage time at 4 °C of tortellini-like Turkish food, for short-term periods (28 days) (Uzunlu 2019).

15.4.2 PVA-Based Food Packaging Materials

PVA is among the few synthetic biodegradable polymers that are frequently used as food packaging material due to its properties, such as flexibility and hardness, solubility in water, transparency, film forming ability, good barrier properties to oxygen and aroma, compatibility, and high tensile strength. Compared with common conventional plastics used as food packaging materials, such as PET or LDPE, PVA is a high cost polymer. It is known that partially hydrolyzed PVA is used as an active food packaging material for protection of foods from oxygen, moisture, and other environmental contaminants. In 2005, a partially hydrolyzed PVA with an average molecular mass in the range of 26,000–30,000 Da, has been evaluated by the European Food Safety Authority (EFSA) as a food additive used as film coating agent for food supplements. It was found that the use of PVA is of no safety concern in the coating of food supplements that are in the form of capsules and tablets (EFSA Journal 2007).

An active food packaging material based on PVA and natural extracts such as mint and pomegranate peel extract was successfully developed by Kanatt et al. (2012). The tensile strength was improved by the addition of the mint and pomegranate peel extract, the water vapor transmission significantly decreased with increasing chitosan content, and the permeability values showed that all the films were impermeable to oxygen. Moreover, the efficiency in meat preservation of active films based on PVA blends with carboxymethyl cellulose (CMC) and clove oil prepared by solvent casting was demonstrated by Muppalla et al. (2014). It was found that the ground chicken meat samples packed in PVA/CMC/clove oil films exhibited a shelf life of 12 days, whereas meat packed in LDPE pouches (control samples) spoiled within 4 days during refrigerated storage at 4 °C for 16 days. Kanatt and Makwana (2020) developed an eco-friendly, water-resistant active packaging film based on PVA and showed that incorporation of citric acid and aloe vera improved the physicochemical properties of the PVA films and extended the shelf life of minced meat.

It is well known that silver and chitosan nanoparticles are effective antimicrobial agents; hence nanocomposites films based on PVA–chitosan/Ag nanoparticles were obtained (by solvent casting) and tested as wrap package on chicken sausage (Nwabor et al. 2020). The results showed that the addition of *Eucalyptus*

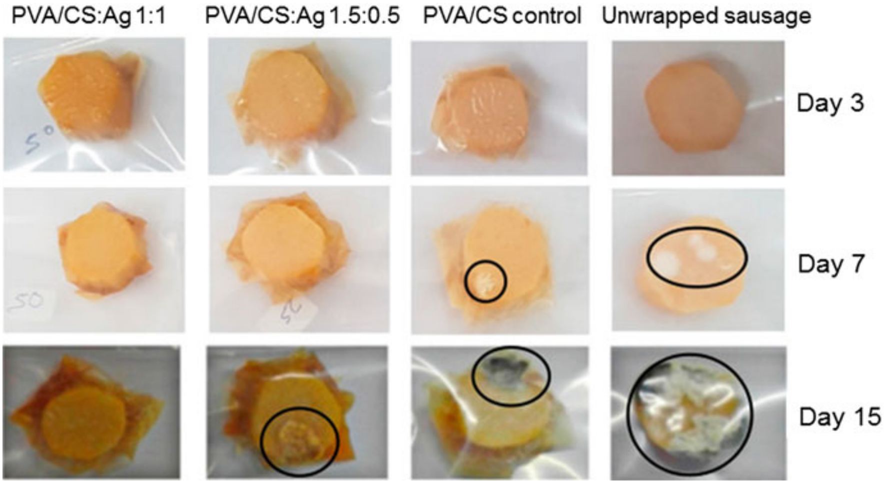


Fig. 15.1 Effects of polyvinyl alcohol-chitosan films incorporating Ag nanoparticles on the spoilage retarding of chicken sausage slices stored at 20 °C. (Reproduced from Nwabor et al. (2020) with permission from Elsevier)

camaldulensis synthesized Ag nanoparticles retarded the spoilage effects over the storage period as compared with PVA/CS control, where coloration and growth of spoilage fungi after 15 days storage can be visually observed (Fig. 15.1).

Electrospinning is another suitable technique to develop active food packaging materials by encapsulation of active agents (Deshwal and Panjagari 2017) and is well demonstrated that PVA forms excellent nanofibers through electrospinning. Lately, electrospinning of PVA is extensively used to encapsulate different active agents (D-limonene, vanillin, essential oils, etc.), because it behaves as a carrier that ensure the sufficient molecular entanglement or as a protective shell. Various electrospun PVA nanofiber mats were developed for active food applications such as carbon dioxide emitter systems (Félix et al. 2016) and antimicrobial or antioxidant materials (Lan et al. 2019; Kayaci and Uyar 2012; Wen et al. 2016) for achieving long-term shelf life of food. An interesting study was realized by Wen et al. (2016) through electrospinning of PVA/cinnamon essential oil/β-cyclodextrin for obtaining an antimicrobial nanofibrous film. It was shown that the PVA nanofibrous film effectively prolonged the shelf life of strawberry, proving that it is suitable for active food packaging application (Fig. 15.2).

15.4.3 PBAT-Based Food Packaging Materials

Various bioactive compounds were incorporated into PBAT to impart different active properties proper for food packaging applications. Ferulic acid (FA) was used as a filler to increase the mechanical and antioxidant properties. FA was incorporated by Sharma et al. (2020) at several concentrations (1, 5, and 10 wt%)

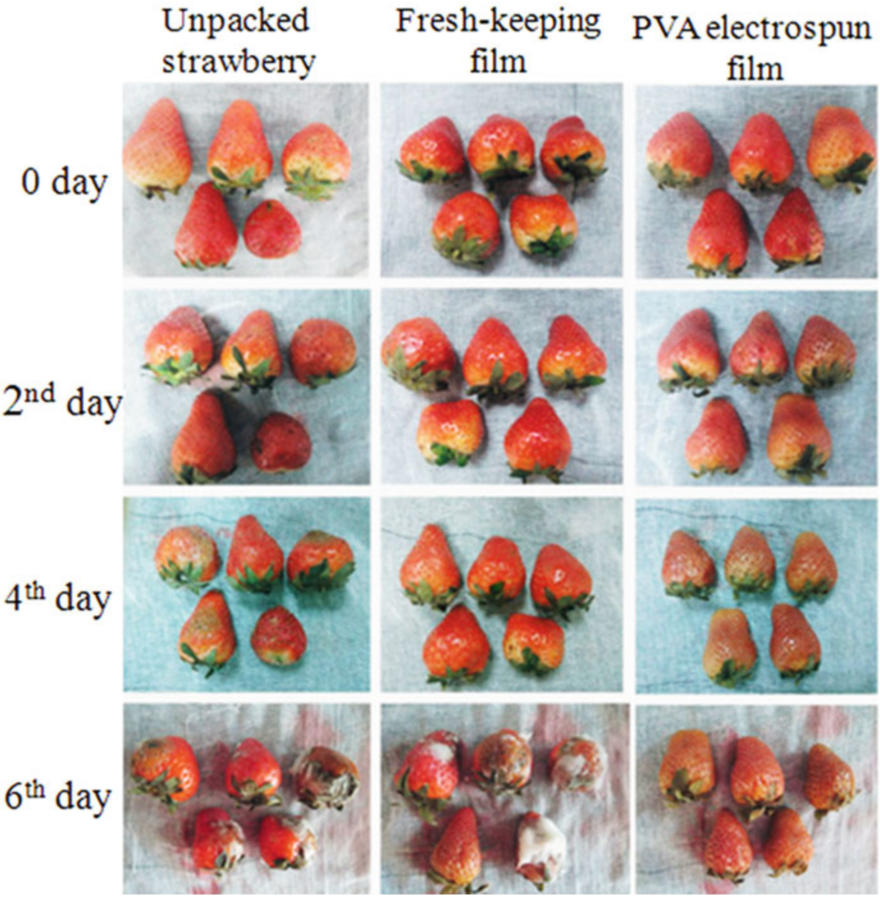


Fig. 15.2 Comparison of visual changes of unpacked strawberries packed with fresh-keeping film and packed with electrospun PVA/cinnamon essential oil/β-cyclodextrin film. (Reproduced from Wen et al. (2016) with permission from Elsevier 2020)

in the PLA/PBAT blend film by solution casting method. It was found that the PLA/PBAT film incorporated with FA has shown high UV-barrier properties, increased mechanical properties, and strong antibacterial activity. Blend films with PLA and PBAT were also prepared using a solvent-casting method by Wang et al. (2016), and packaging test was conducted on fresh green onion and potatoes; it was found that blending with PBAT reduced the brittleness and improved the flexibility of PLA. Also, it was proved that the PLA/PBAT films can extend the shelf life of fresh fruits and vegetables. More examples of active packaging systems based on synthetic biodegradable plastics are listed in Table 15.1.

Table 15.1 Recently developed active packaging materials based on synthetic bio-plastics

Bio-plastic	Type of active component	Obtaining procedure	Type of tested food	Functions of active packaging	References
Poly(caprolactone)/chitosan	Chlorogenic acid loaded halloysite nanotubes	Electrospinning	Antimicrobial testing	Antibacterial and antioxidant Increasing shelf life	Zou et al. (2020)
Poly(caprolactone)	Acetylated nanocellulose	Solvent casting PCL coating	Antioxidant, mechanical, water contact angle testing	Water barrier	Mugwagwa and Chimphango (2020)
Chitosan/poly (caprolactone)	Nanocellulose Grape seeds extract	Solvent casting	Antimicrobial, antioxidant testing	Antioxidant, antimicrobial, water vapor permeability	Sogut and Seydim (2018)
Poly(lactide)/poly (ethylene glycol)/poly (caprolactone)	Zinc oxide, clove essential oil	Solvent casting	Scrambled egg	Microbial inactivation	Ahmed et al. (2019)
Poly(caprolactone)	Sodium metabisulfite	Melt compounding and melt-extruding	Apples	Anti-browning Antimicrobial, Antioxidant, antifungal	Jeong et al. (2020)
Poly(caprolactone)	Equilibrium-modified atmosphere packaging	Twin-screw extruder	Strawberries	Enhanced the shelf life	Yun et al. (2017)
Poly(vinyl alcohol)/carboxymethyl cellulose	Clove oil	Solvent casting	Chicken meat	Shelf life extension	Muppalla et al. (2014)
Poly(vinyl alcohol)/carboxymethyl cellulose/	Aloe vera gel	Solvent casting	Chicken meat	Shelf life extension	Kanatt and Makwana (2020)
Poly(vinyl alcohol)/gelatin	Amaranthus leaf extract	Solvent casting	Fish/chicken meat	Visual monitoring	Kanatt (2020)
Poly(vinyl alcohol)/β-cyclodextrin	Cinnamon essential oil	Electrospinning	Strawberries	Antimicrobial, shelf life extension	Wen et al. (2016)

Poly(vinyl alcohol)	Montmorillonite clay/ginger extract/silver nanocomposites	Solvent casting	Chicken sausages	Antibacterial, shelf life extension	Shiji et al. (2019)
Poly(vinyl alcohol)	Cinnamon essential oil	Electrospinning	Raw shrimps	Antibacterial, shelf life extension	Nazari et al. (2019)
Poly(vinyl alcohol)/ chitosan	Ag nanoparticles with ethanolic leaf extracts of *Eucalyptus camaldulensis*	Solvent casting	Chicken sausage	Antimicrobial, antioxidant shelf life extension	Nwabor et al. (2020)
Poly(butylene adipate-co-terephthalate)	Poly(lactic acid)	Solvent casting	Fresh green onion and potatoes	Shelf life extension	Wang et al. (2016)
Poly(butylene adipate-co-terephthalate)	Oregano essential oil	Hot-melt extrusion	Fish fillets	Shelf life extension up to 10 days	Cardoso et al. (2017)
Poly(butylene adipate-co-terephthalate)	TiO_2/Ag nanoparticles	Solvent casting	Cherry tomato	Mechanical, barrier, antimicrobial	Cao et al. (2020)
Poly(lactide)/poly (butylene adipate-co-terephthalate)	Ferulic acid	Solvent casting	Antibacterial test	Antibacterial, UV-light barrier	Sharma et al. (2020)

15.5 Conclusion

Significant advancement has been attained in finding and optimizing synthetic bioplastics with suitable properties in food packaging. Reported studies on synthetic biodegradable plastics applied in active food packaging includes polymers with hydrolysable functions such as poly(caprolactone), poly(butylene adipate-co-terephthalate), or polymers with carbon backbones that require an oxidation process prior biodegradation, such as poly(vinyl alcohol). At this time, these bioplastics are still far from being an economical and feasible alternative to compete with conventional plastics. Moreover, the active packaging domain is still evolving, and a lot of research is needed to facilitate technology upscaling.

Acknowledgments This work was supported by grants of the Romanian Ministry of Research, Innovation and Digitization, CNCS/CCCDI—UEFISCDI, project number PN-III-P1-1.1-PD-2019-1101 number PD 31/2020 and PN-III-P1-1.1-PD-2019-1120 number PD 49/2020, within PNCDI III.

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Bioplastic Matrices for Sustainable Agricultural and Horticultural Applications

16

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Abstract

Bioplastics have become a potential alternative to the use of plastics. More specifically, they will contribute to reducing the high accumulation of plastics, which is caused by their low biodegradability. In addition, these bioplastics can be obtained from waste and by-products from other industries (mainly agri-food), which not only lower their costs but also allow them to make the most available resources, contributing to support the circular economy. In the agriculture and horticulture sectors, these bioplastics provide new alternatives that reduce the use of resources. Thus, superabsorbent bioplastics can be used as a complementary water source, since they can absorb and retain a large amount of water, supplying it to crops in drought times. In this way, the water cycle is improved, by rationalizing crop irrigation. Moreover, these bioplastics can also be used for the controlled release of nutrients and can be modulated with their biodegradability. Thus, their assimilation efficiency by the crops is improved, avoiding the excessive use of fertilizers. However, to achieve these benefits, it is essential to study the processing of these materials to have the required functional characteristics. Therefore, this chapter evaluates the increase use of bioplastics in the agricultural and horticultural sectors, paying great attention to the raw materials used in these bioplastics, their processing method, characterization, and potential applications. In this sense, current and future trends in this field also have been reported and compared.

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Keywords

Agriculture · Bioplastics · Controlled release · Horticulture · Water source

16.1 Introduction

Nowadays, plastics are the most used materials in daily life. They can be found in many varied applications, e.g., food packaging, automotive parts, electronic elements, decoration, textile, agriculture, and toys. Consequently, the production of plastic increases every year, reaching 357 million tons in 2019 (Plastics Europe 2020). However, their use presents a problem derived from their low biodegradability (Hassan and Haq 2019): their half-life is too long (sometimes up to thousands of years) compared to their shelf life, which can be from a few seconds in packaging to 5–10 years in more sophisticated applications such as electronics. This would not be a problem if this material could be reused or recycled correctly; however, only 10% of plastics had this destination in 2019, with the majority being disposed of in landfills or simply in nature (Hopewell et al. 2009). This low reuse and recycling of plastics has generated a problem of accumulation, which ultimately affects the terrestrial ecosystem, as well as the aquatic and aerial ecosystems (Sheavly and Register 2007; Allen et al. 2020). Furthermore, the production of these plastics generates a high carbon footprint, mostly due to their petroleum origin, which contributes to increasing global warming (Dormer et al. 2013). All these problems have led more and more people to become aware of the responsibility that the use of plastics entails, considering their reduction through alternative materials (Maldonado et al. 2014). In this context, wood, glass, and even some metals, such as aluminum, are increasingly used in applications where only plastics were previously used. However, due to their higher price and their limitations in some applications, such as agriculture and horticulture, conventional plastic continues to be the most widely used material, as can be seen in Fig. 16.1, which compares the different materials used in several applications in 2019.

A tentative alternative that has shown better results in the substitution of plastic is the use of bioplastics. The definition of bioplastic has two connotations; thus, it can refer to (1) plastics from renewable sources or (2) biodegradable plastics (Remar 2011). However, the most interesting bioplastics are those that bear both connotations, i.e., bioplastics that come from natural and renewable sources, which does not limit their existence and decreases their carbon footprint, while they are also biodegradable, reducing the pollution generated by plastics (Dhillon et al. 2016). In addition, bioplastics can have similar mechanical and functional properties than conventional plastics, so they are potential candidates to replace them, being better welcomed than the other proposed alternatives. In this way, the use of bioplastics has increased notably in the last years, although the growth in the use of plastics has slowed down (Fig. 16.2). It suggests that much more research is necessary for bioplastics to replace conventional plastics to a greater extent (Plastics Europe 2020).

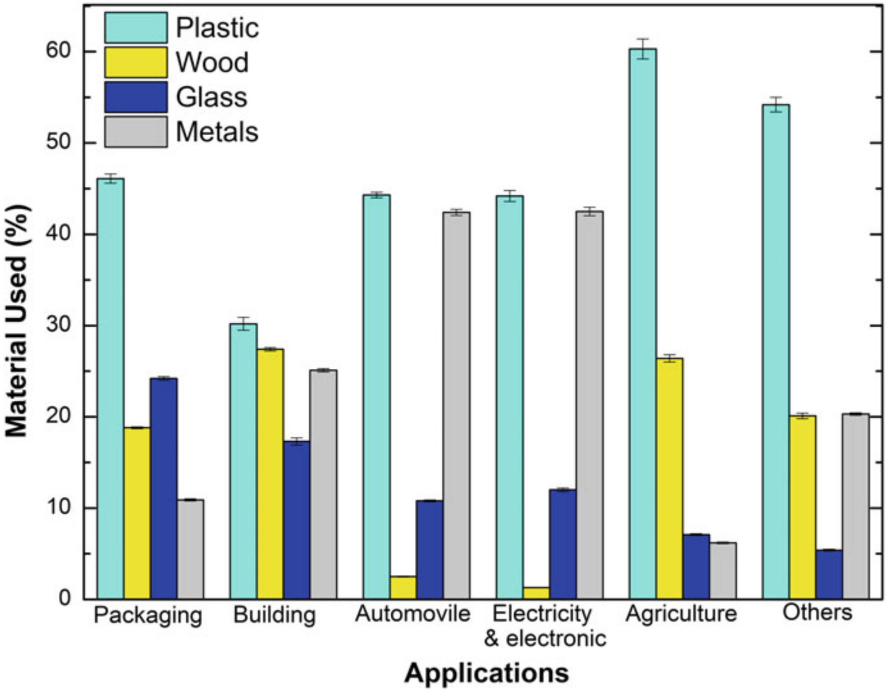

Fig. 16.1 Use of materials in different industrial sectors in 2019

These bioplastics could be manufactured from agri-food wastes or by-products, cellulose, starch, proteins, or fibers (Yadav et al. 2020). The structure of these raw materials is similar to that of conventional plastic. In this way, they allow process bioplastics with the common manufacturing method used in plastics, such as injection molding, extrusion, and compression molding. Thus, several conventional methods can be adapted to produce bioplastics. In this way, the initial economic investment for the elaboration of these products is reduced. Furthermore, bioplastics can be processed with more sophisticated techniques, such as fermentation, electrospinning, or 3D printing (Tsang et al. 2019). All these advantages of bioplastics and the similarity between plastic and bioplastics are not going unnoticed, as many industrial sectors are benefiting from them (Neila 2016). Among these industrial sectors, agriculture and horticulture are the sectors that are investing the most in the use of bioplastics. Agriculture is a set of technical and economic activities related to soil treatment and the cultivation of land for human consumption (Keeney 1990). Among its branches, horticulture oversees growing vegetables, fruits, and herbaceous plants (Relf 1992). Although agriculture and horticulture were not considered a normal practice at their beginning, since they were related to low social status, their interest has increased throughout history. Thus, their products are increasingly demanded, being viewed as essential products for a healthy diet (Maroto 2008). For example, this increase can be observed in potato production

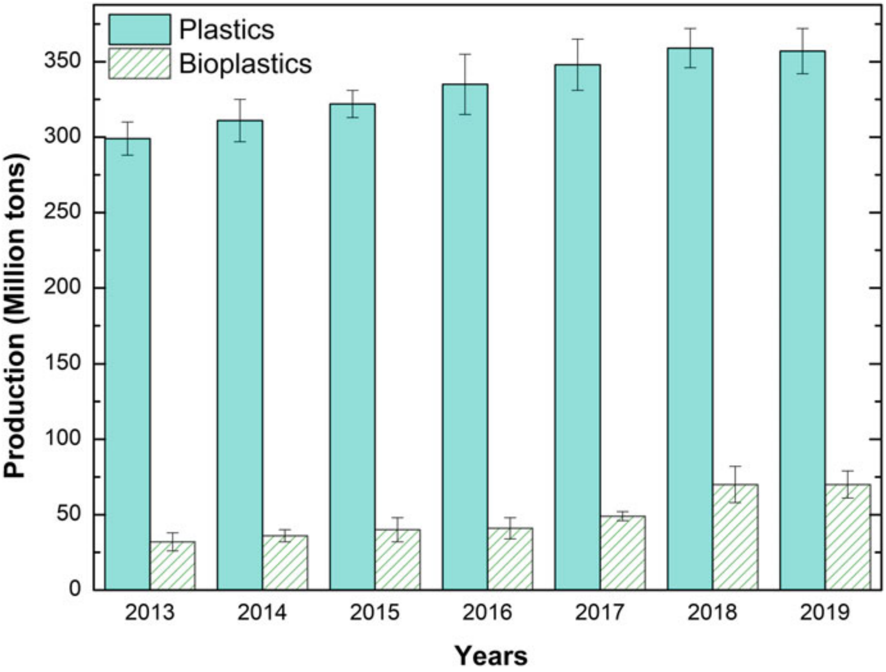

Fig. 16.2 Plastic and bioplastic production in different years

(Fig. 16.3a), which is the most demanded agricultural product. In this sense, potato production has increased by 24% in the last 20 years. Regarding where it is produced, Asia holds the first place, followed by Europe (Fig. 16.3b). This is due to the greater cultivation area available on these continents, which is combined with the ideal climatic conditions for their production (Food and Agriculture Organization of the United Nations 2019). This increase in production has generated a new way of perceiving agriculture, opting for intensive cultivation to meet the demand (Hazell and Wood 2008). This intensive agriculture, together with the increased presence of pests and changes in weather conditions (due to climate change), has caused an increase in the use of plastic devices in this sector, including devices to supply water or fertilizers and to protect the crop from weeds or insects. Nevertheless, the use of these plastics has an extra cost in the products, since it is not only necessary to pay for their purchase, but it is also required to pay for their removal, once their work is done, to prevent the contamination of the soil (Karan et al. 2019). In this way, the use of bioplastics can solve these problems due to their biodegradability, without the need to collect them after their use. Furthermore, their raw materials can be supplied from cheaper sources, making the cost of the final bioplastics cheaper than conventional plastics (Palani and Karthikeyan 2020). All this comes down to the fact that the plastics used in agriculture is increasingly replaced by using bioplastics, as seen in the research conducted on both materials in agriculture and horticulture (Fig. 16.4).

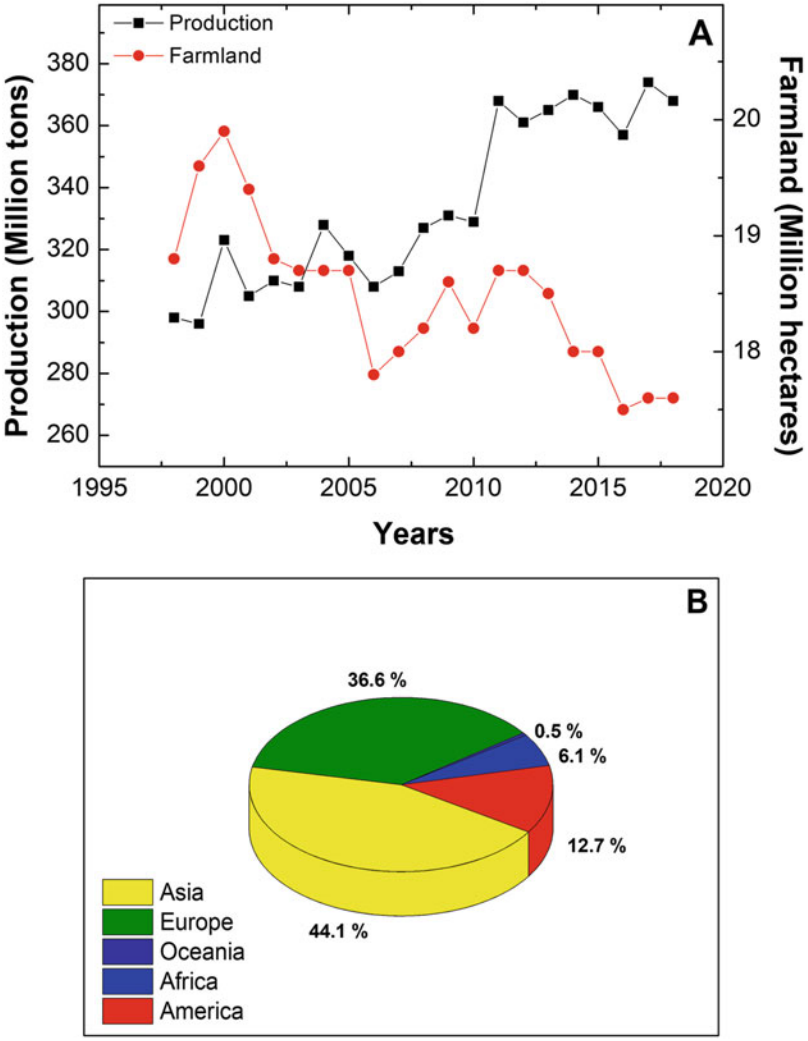

Fig. 16.3 Global potato production and farmland (**a**) and production by continents (**b**) from 1998 to 2018

In this context, the objective of this chapter is to highlight the use of bioplastics in agriculture, explaining the applications where they are most demanded, as well as the raw materials and processing method used to obtain these bioplastics. In addition, the necessary characterization to evaluate the use of these bioplastics in agriculture and horticulture is also explained.

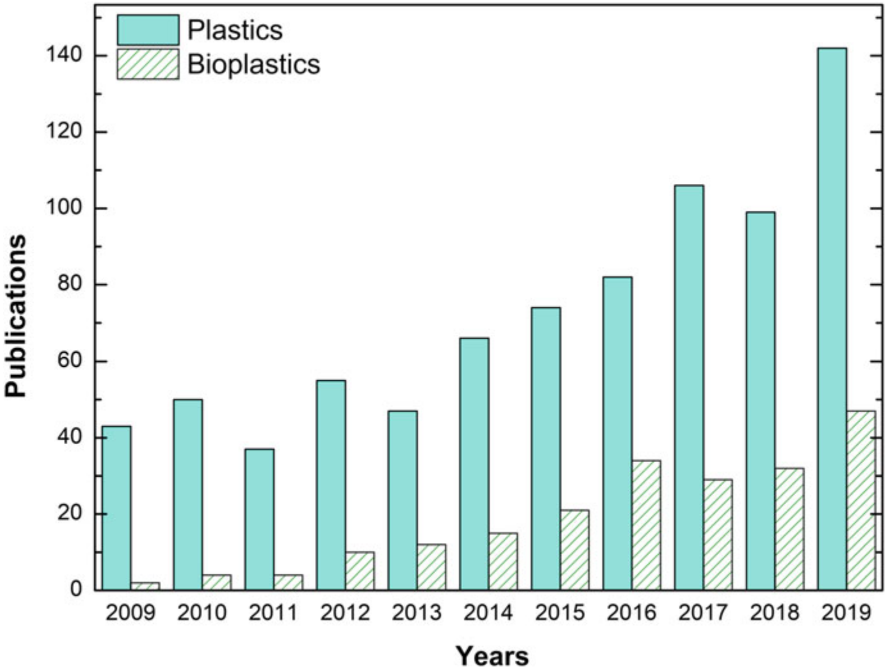

Fig. 16.4 Publications for plastics and bioplastics in agriculture and horticulture from 2009 to 2019 (Data from Web of Science)

16.2 Bioplastic Matrices in Agricultural and Horticultural Applications

More and more bioplastics are used in agriculture and horticulture applications. Normally, these bioplastics are used as matrices that support different applications, such as retaining water, fertilizer, or pesticides. However, there are other applications where bioplastics are not only utilized as matrices, but also as protection bags against weeds or insects.

16.2.1 Bioplastic Matrices as a Supplementary Water Source

An irrigation system is becoming necessary in more areas due to the long periods of drought-induced by climate change (Thiery et al. 2020). However, in many areas, this irrigation is very expensive or even impossible to install because nearby water sources are not available (Easter 2019). In this context, some commercial plastics have been developed in order to supply water to crops in a simpler manner, such as Creasorb by Evonic or Luquasorb by BASF. These plastics can capture a large amount of water during rainy periods, even if they are scarce, and then they can supply water gradually to deliver the plant needs. However, these plastics have only one use, so they must be removed once they have fulfilled their purpose to prevent them from transmitting toxic substances to the food products, which poses an additional cost to the use of these systems. In this sense, the use of bioplastic matrices could be a solution. Some of these bioplastic matrices can be considered superabsorbent; that is, they can retain 1000% of their weight in water without disintegrating (Cuadri et al. 2017). These superabsorbent bioplastic matrices can capture water like the previously mentioned plastics and supply it during their biodegradation; thus, it is not necessary to remove them from the crop at the end of their use. Therefore, these systems are gaining interest in this sector. Different bioplastic matrices are being investigated, where the absorbent capacity of bioplastic matrices is maximized without damaging their mechanical properties and biodegradability, in order to achieve sustenance of water throughout the growth of the crop (Gómez-Martínez et al. 2009; Cuadri et al. 2016; Álvarez-Castillo et al. 2019, 2020a; Fernández-Espada et al. 2019; Jiménez-Rosado et al. 2019a; Olad et al. 2020).

16.2.2 Bioplastic Matrices as Devices for the Controlled Release of Fertilizers

The controlled release of fertilizers is the latest application for bioplastic matrices in the agriculture and horticulture sectors. Their research comes from the recent awareness of the damages in the subsoil and groundwater caused by the excess of fertilizers used in crops (Kondraju and Rajan 2019). Thus, different alternatives have been assessed, such as the use of conservation tillage or the prevention in the use of anthropogenic residues, but the versatility and speed that fertilizers generate in the crops make these alternatives not competitive (Stinner and House 1990; Derraik 2002). The latest trend is to study systems where fertilizers can be released in a controlled way, to adapt them to the needs of crops, and, thus, to improve assimilation efficiency, reducing the pollution generated. In this context, different plastic systems have been industrially commercialized (Nutricote by Projar and Multicote by Haifa), but they present problems derived from the low biodegradability of the used plastics, which remain in the soil and are difficult to remove. For this reason, bioplastic matrices are attracting attention in this application. These bioplastic matrices can support the fertilizers as filling material. Later, the fertilizers can be released in a controlled manner through the irrigation water or through the biodegradation of the bioplastic matrix (Mortain et al. 2004). Thus, fertilizer efficiency can be improved, and the problems derived from removing the systems are eliminated, since they degrade into non-toxic products for cultivation (Karan et al. 2019). In this context, different studies are evaluating the proportion of different nutrients that can be incorporated into bioplastic matrices (Pereira et al. 2015; Jiménez-Rosado et al. 2018, 2020a; de Souza et al. 2018; Merino et al. 2019; Mesias et al. 2019). Furthermore, researchers are studying how to adjust the kinetics of nutrient release to the need of each crop, with the aim of obtaining more specialized fertilization, improving its yield.

16.2.3 Other Applications

There are other applications where bioplastics can be used in agriculture and horticulture. For example, bioplastics can be used as traps for insects and microorganisms to avoid the use of pesticides (Merino et al. 2018). This practice is very attractive in organic crops, as it prevents plants from being damaged by the use of toxic substances. However, it is necessary to incorporate some additives in order to improve the attraction of microorganisms and insects to these materials. For this, natural substances that present attractive odors are usually used for them, such as imidacloprid (Singh et al. 2020). These substances can be released in a controlled way through the biodegradation of bioplastics, making the systems effective for a long period of time.

Another application for these bioplastics is their use as padding. These materials protect soils from excessive evaporation and competing plants, without resorting to excessive tillage (Elbasiouny et al. 2020). Different materials such as straw, cardboard, gravel, or plastics are already being used for these applications since they improve crop yields (Amidor 2019). However, some works have demonstrated that the use of bioplastics in this application further improves crop yield due to less nitrate loss, higher water use efficiency, and higher water vapor permeability (Elbasiouny et al. 2020). Although the price of bioplastics is usually higher for this application, many governments subsidize their use, since, apart from improving performance, it generates less pollution to the environment (Cosma 2018). Thus, they are increasingly implemented. Moreover, due to their mentioned properties, these bioplastics are widely used in ecosystems where the environmental conditions are extreme (very hot or very cold) since they allow cultivation by preserving the plants in optimal temperature and humidity conditions, avoiding excessive import of these products from other zones.

Lastly, bioplastics can be used as seedbeds and plant guides, which do not need to be removed after use, avoiding problems derived from cutting or damaging the roots or stems of the crops (Mosnáčková et al. 2019). However, for these applications, and those previously mentioned in this section, bioplastics must hold good mechanical properties throughout their use, which are sometimes affected by their biodegradability. Therefore, an exhaustive study of all conditions is necessary for their optimal use.

16.3 Fabrication of Bioplastic Matrices

The most important factors that can modify the properties of the processed bioplastics are the raw materials employed and the processing methods applied. Thus, there is a wide variety of properties that can be obtained by modifying these factors.

16.3.1 Raw Materials

Generally, bioplastics are composed of a biopolymer, a plasticizer, and, sometimes, additives (Saenghirunwattana et al. 2014). The biopolymer forms a matrix that provides the plastic character to the material. Usually, it is composed of proteins and/or polysaccharides due to the similarity of their chains with those of conventional plastics (Zárate-Ramírez et al. 2014). The choice of material for the bioplastic matrix will depend on its final application. Thus, if the bioplastic matrix is intended to retain large amounts of water, the raw material should have hydrophilic groups that can interact with water molecules, facilitating water uptake and retention. In this context, the high content of glutamic and aspartic acids in some proteins favors the absorption of water (Fernández-Espada et al. 2016a). Among polysaccharides, acrylic monomers are often chosen, with polyacrylates being the most extensively selected to develop high-performance superabsorbent materials (Liu et al. 2019). On the other hand, if the bioplastic matrix is intended to retain fertilizers or pesticides, the raw material should have good covalent interactions between the biopolymeric chains and interconnection with the added fertilizers and pesticides, so that bioplastic matrices have good mechanical properties and release the substances in an appropriate controlled way during their biodegradation (Jiménez-Rosado et al. 2020a). Finally, if the bioplastic matrix is used as padding, seedbeds, or plant guides, it needs good mechanical properties to prevent it from being harmed during its use, followed by rapid biodegradation. This requires a good strengthening between the biopolymeric chains (Félix et al. 2014). In this context, raw materials with a high protein or polysaccharide content are used to facilitate interactions between the chains of the biopolymeric matrix, such as isolates.

However, sometimes the biopolymeric matrix is not enough to fulfill the mentioned purposes, so it is necessary to incorporate additives to improve their properties. Additives are commonly used to improve the mechanical properties of bioplastic matrices and, thus, to enhance strengthening properties (Felix et al. 2017). Fibers are the most investigated elements in this context due to their high tensile and flexural properties (Saenghirunwattana et al. 2014; Lubis et al. 2018). Nevertheless, crosslinking agents, such as citric acid, are sometime used (Awadhiya et al. 2016). However, it is difficult to find a natural and biodegradable additive that guarantees a sufficient reproducibility of mechanical characteristics, with cellulose-based fibers being the most reliable and, thus, the most used. Other additives for the improvement of mechanical properties are nanoclays and nanoparticles, although it is necessary to check that these additives do not affect the plant, since due to their small size they can be absorbed by plants, causing food poisoning (Perez-Puyana et al. 2018). It should be noted that all the additives used to strengthen bioplastic matrices for agriculture and horticulture must have a similar biodegradability to that of the matrices so as not to leave residues that can accumulate in the cultivation soils. However, additives are not only used to improve the mechanical properties of bioplastic matrices, but they are also necessary for applications such as the controlled release of fertilizers and pesticides. For fertilizers, the same salts used in conventional fertilization are usually used, since these have the necessary nutrients for plants (Mesias et al. 2019; Jiménez-Rosado et al. 2020a), although it must be taken into account that the biodegradation of bioplastic matrices also provides nutrients to the crop, such as nitrogen and carbon. As for pesticides, natural substances that are not harmful to plants and the environment are normally employed (Neri-Badang and Chakraborty 2019). The use of one substance or another depends on the pests that affect each crop, with citronella and zinc oxide nanoparticles being the most recently investigated in this field (Djiwanti and Supriadi 2019; Khan and Pathak 2020). Even so, the interactions between these components (fertilizers or pesticides) and the bioplastic matrix must be weak. The importance of these interactions is crucial, since, if they are too strong (i.e., covalent bonds), the substances cannot be released as the crops need it, whereas, if the interactions are non-existent, the bioplastic matrix will not be able to act as a controlled dispensing device, producing an unwanted quick release (Jiménez-Rosado et al. 2020a). In this sense, the interactions that are sought are electrostatic, which are achieved by displacing the pH of the bioplastic matrix so that it is not at its isoelectric point (Jiménez-Rosado et al. 2020b).

Plasticizers are required to improve the processability of bioplastic matrices (ASTM D883-00 2000). A plasticizer is a substance that is incorporated into a material to increase its flexibility and facilitate its transformation. Thus, it can reduce the viscosity of the blends, lower the glass transition temperature, and/or decrease the elastic modulus of the blend. It must be considered that for the application in agriculture and horticulture, there are two possibilities concerning the fertilizer used: (1) using natural plasticizers that are not harmful to the environment and (2) eliminating the plasticizer after processing the matrix and before its final use (Jiménez-Rosado et al. 2018). Water is the plasticizer that best complies with these two conditions. In this sense, it holds the ability to reduce the glass transition temperature and increase the free volume of biopolymers, as well as being abundant and cheap. However, its use as a plasticizer in bioplastic matrices worsens their mechanical properties, making the use of other substances more convenient (Matveev et al. 2000). In this context, the most common plasticizers are polyols and oligosaccharides, such as glycerol, ethylene glycol, diethylene glycol, and mannitol (Aguilar et al. 2020).

Table 16.1 summarizes the raw materials used in each of the mentioned applications. There are raw materials for biopolymeric matrices that are used in various applications, and they are processed differently to enhance some properties or others. As can be seen, proteins have been used more frequently than polysaccharides in this area, due to the better mechanical and functional properties of the former (Ryder et al. 2020). However, the combined use of proteins and polysaccharides is being investigated lately (Gutiérrez 2018). These composite systems show synergies between both biopolymers, improving the properties that both have separately. Thus, there are systems combining sago starch and protein flour. As for plasticizers, they are generally used in all applications. However, it is worth mentioning that in applications where a controlled release of additives (fertilizers and pesticides) is desired, the plasticizer must be removed before use, since most plasticizers are very similar to water, causing them to be released into the

Table 16.1 Raw materials and processing methods used to manufacture bioplastic matrices for different agricultural and horticultural applications

Applications	Raw materials			Processing method	References
	Biopolymeric matrix	Plasticizer	Additive		
Supplementary water source	Porcine plasma protein	Glycerol	–	Mixing + injection molding	Álvarez-Castillo et al. (2019), Álvarez-Castillo et al. (2020b)
	Wheat gluten	Glycerol, water	KCl, citric acid	Mixing + compression molding	Gómez-Martínez et al. (2009)
	Potato starch	Basic water (NaOH)	Cellulose nanocrystals	Freeze-drying	Olad et al. (2020)
	Soy protein isolate	Glycerol	–	Functionalization + mixing + injection molding	Cuadri et al. (2016)
		Glycerol	Sodium bicarbonate, sodium carbonate, Zn-AEDT	Mixing + injection molding	Fernández-Espada et al. (2019), Jiménez-Rosado et al. (2019a, b)
Devices for the controlled release of fertilizers	Soy protein isolate	Glycerol	Zinc sulfate, zinc perchlorate, zinc carbonate	Mixing + injection molding (+plasticizer removal + lyophilization)	Jiménez-Rosado et al. (2018, 2020d)
	Corn starch	Glycerol, water	Sodium tripolyphosphate, potassium sulfate	Manual mixing + extrusion	Merino et al. (2019)
	Polycaprolactone	Water	Urea, montmorillonite clay	Mixing + extrusion + drying	Pereira et al. (2015)
	Starch + polyhydroxybutyrate	Glycerol	Sodium montmorillonite clay, potassium nitrate	Mixing + compression molding	de Souza et al. (2018)

(continued)

Table 16.1 (continued)

Applications	Raw materials			Processing method	References
	Biopolymeric matrix	Plasticizer	Additive		
	Chitosan + sodium alginate	Acidic water (acetic and citric acid)	NPK	Mixing + chemical crosslinking	Mesias et al. (2019)
	Carboxymetyl cellulose	Water	NPK	Solvent casting	Kartini et al. (2020)
Other applications	Soy protein isolate	Glycerol Ethylene glycol, diethylene glycol, triethylene glycol	Nanoclay, lignocelullose	Mixing + injection molding + (heat treatment or ultrasound treatment)	Bourny et al. (2017), Gamero et al. (2019), Aguilar et al. (2020), Jiménez-Rosado et al. (2020a)
	Pea protein isolate	Glycerol	–	Mixing + injection molding	Perez-Puyana et al. (2017)
	Wheat gluten	Glycerol, water	Xanthan gum, glyoxal	Extrusion	Jiménez-Rosado et al. (2019a, b)
	Wheat gluten	Glycerol, water	locust bean gum, methyl cellulose, carboxymethyl cellulose, formaldehyde, glutaraldehyde, glyoxal	Mixing + compression molding	Zárate-Ramírez et al. (2014)
	Sago starch + protein flour	Glycerol	–	Mixing + compression molding	Gutiérrez (2018)
	Corn starch	Glycerol	Natural bentonite, chitosan-modified bentonite	Extrusion	Merino et al. (2018)
	Canola protein meal	Glycerol	Cellulose fiber	Mixing + extrusion + compression	Li et al. (2019)
	Cassava	Glycerol + water	Vegetable oil	Casting	Isroi et al. (2018)

environment during irrigation, uncontrollably dragging additives (Jiménez-Rosado et al. 2020c). Finally, the additives depend on the application, since these are used to improve a specific property of the bioplastic matrix. Nevertheless, as can be seen, their inclusion is not always necessary.

16.3.2 Processing Methods

One of the positive points of the elaboration of bioplastic matrices from biopolymers is the easy adaptation of the processing methods used for the elaboration of conventional plastics to produce bioplastic matrices (Fakirov et al. 2007; Chantapet et al. 2013). This reduces the investment made for the development of these systems, causing many producers to adhere to government subsidies to adapt their factories for the production of bioplastic matrices (Pirzada et al. 2020). The most used processing methods are compression molding, extrusion, and injection molding. All of them coincide in the fundamental stages: the rupture of intermolecular bonds that stabilize biopolymers to give them mobility, rearrangement and orientation of the mobile chains of the polymer in the desired form, and, finally, the formation of new intermolecular bonds and interactions that allow the stabilization of the originated three-dimensional matrix. Although there are also other highly studied methods, such as casting, their worse assimilation to already used processes makes them economically unprofitable for agricultural or horticultural applications, being used in sectors where the cost of the final product is less important, such as cosmetics (Guilbert and Gontard 2005). As can be seen in Table 16.1, all of them are regularly used for the processing of bioplastic matrices, but, in most cases, it is necessary to incorporate additional steps to improve the process.

16.3.2.1 Compression Molding

Compression molding uses a combination of pressure and temperature to process the samples. Heating biopolymers above the glass transition temperature makes the material easily moldable, although polymerization can also occur due to denaturation of the biopolymer, depending on the processing conditions and the nature of the plasticizer (Jerez et al. 2007). In this technique, the material is placed in a mold where heat and pressure are applied so that it meets the entire surface of the mold, adapting to its shape. These conditions are maintained until the material has cured, or the pressure is simply maintained while the material cools and hardens (Fig. 16.5) (Engel et al. 2020). The main advantages of this processing method are its low costs (operation, maintenance, manufacture of molds, etc.) and the possibility of achieving a good surface finish with little waste. However, the curing time can be long (Engel et al. 2020). Normally, it is advisable to use isostatic compression so that the properties of the bioplastic matrix are homogeneous throughout the system (Greene 2014). However, this increases the cost of the processing method, making it unprofitable in many cases.

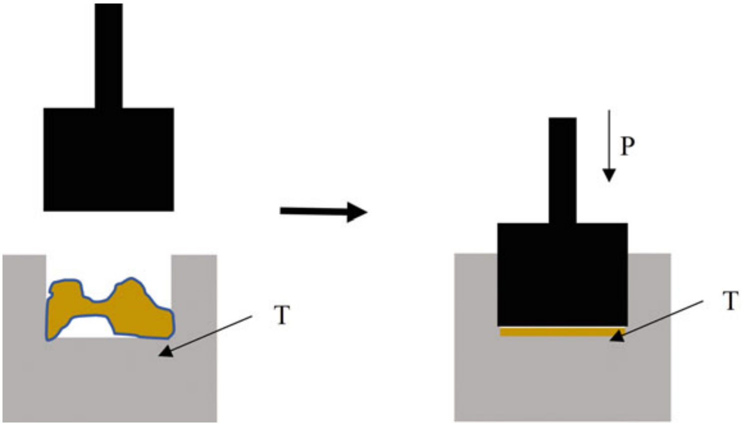

Fig. 16.5 Scheme of compression molding processing method

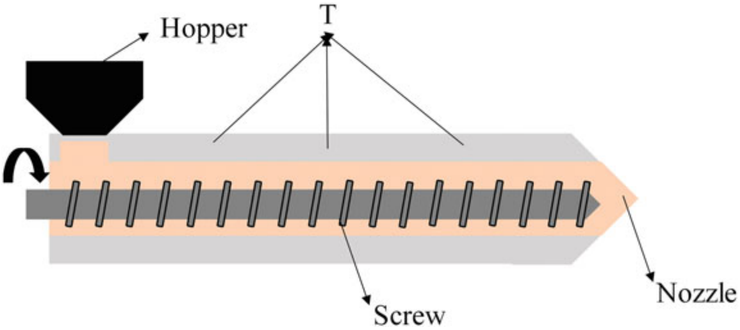

Fig. 16.6 Scheme of extrusion processing equipment

16.3.2.2 Extrusion

This processing method is based on the mixing of protein and plasticizer to obtain a homogeneous blend. Extrusion forces the thermoplastic material, at a suitable temperature, to flow through a nozzle or die, with a more or less complex shape and fixed section, by applying pressure (Ebrahimi et al. 2019). The extrusion equipment is mainly made up of the elements shown in Fig. 16.6. Thus, it consists of a hopper through which the raw material is introduced, a motor that supplies the mechanical energy necessary to transport the material, an extrusion screw that drives the material through the nozzle, and an extruder cylinder that hosts the worm screw and to which the nozzle is attached. The cylinder is provided with different heating elements (electrical resistances or heating jackets) that maintain the appropriate thermal profile for extrusion (Hernandez-Izquierdo and Krochta 2008). Among the main advantages of this processing technique are its high productivity since it operates under continuous steady conditions, its simplicity of operation (not needing a previous homogenization stage of the raw materials), and its moderate costs.

However, it is restricted to products of constant cross-section and usually requires the application of post-extrusion processes. Precisely, the application of this processing method with screws of large kneading blocks generates greater shears on the blends, obtaining bioplastic matrices with good mechanical properties (Graiver et al. 2004).

The temperature of the chamber must reach the glass transition temperature for the blend to flow correctly. Then, a proper temperature profile must be defined throughout the chamber. This is a key point in extrusion processing, taking into account that a certain crosslinking degree among polymer chains may contribute to hold the shape and improve the mechanical properties of the extruded bioplastic. On the other hand, it is essential to prevent excessive crosslinking of the biopolymeric blend to ensure its correct flow through the nozzle (Jiménez-Rosado et al. 2019b).

16.3.2.3 Injection Molding

Like compression molding, injection molding aids in the combined application of pressure and temperature to form the bioplastic matrix. Injection molding is a discontinuous processing technique that involves the application of pressure to fill a mold and the subsequent cooling and demolding of the sample (Adamy and Verbeek 2013). The control of the melting temperature is considered the most critical factor in this type of molding, as it directly affects the viscosity of the molten biopolymer, thus affecting the injection speed, the piston lowering speed, the cooling speed of the mold, and the pressure-time profile (Zárate-Ramírez et al. 2014). The simplest equipment used for injection molding (plunger-type) is shown in Fig. 16.7, consisting of several devices: the cylinder, equipped with heater elements, where the blend is plasticized; the injector system (with the plunger), which is in charge of

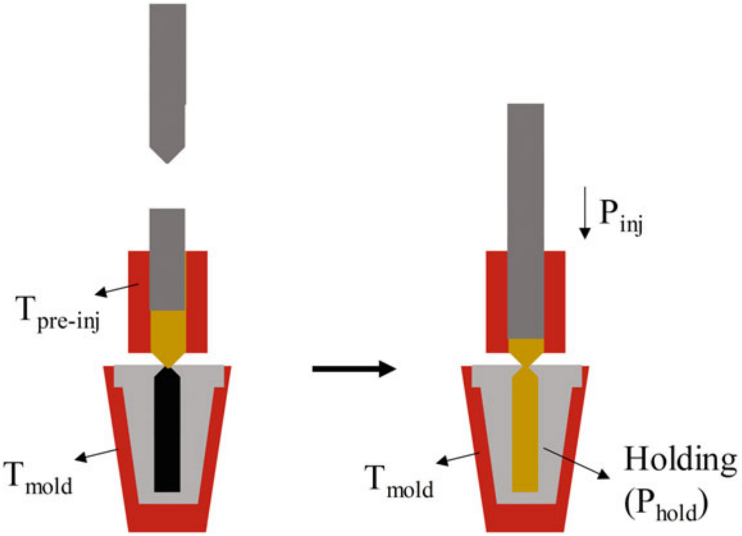

Fig. 16.7 Scheme of a vertical plug-type injection molding equipment

injecting the blend; the closing unit, which contains the mold and the part ejection system; and the control system, which controls the process parameters (time, temperature, pressure, and speed). Industrial large-scale injection molding operations are generally carried out using horizontal screw type machines, where the screw has a double function: firstly, to plasticize the blend inside the cylinder and, secondly, to inject the polymeric material into the mold.

The main advantage of injection molding is that pieces of quite different sizes and shapes, even with complex geometries can be obtained in a single step. Furthermore, it is the most common and most developed processing method in terms of polymer processing, since it is the most economically profitable technique, provided the production scale is large enough. However, it is a complex process that must be optimized to avoid defects in the injection-molded product. In some cases, a pretreatment is required to homogenize the raw materials (Khosravani and Nasiri 2020).

All the mentioned processing methods are suitable for the manufacture of bioplastic matrices, and none of them has proved to be more prominent than the others in the recent literature. However, injection molding is particularly suitable, since the biopolymers can present both thermoplastic and thermosetting characteristics (depending on the processing conditions); therefore, an adequate selection of the processing parameters is essential to obtain the desired properties (Félix et al. 2014; Perez-Puyana et al. 2016). In addition, it is among the most commonly used techniques for synthetic polymer processing, which opens up the availability of many different sizes and types of machines. Nevertheless, this process confers irreversible structural changes to the biopolymers so they cannot be reprocessed (Félix et al. 2014).

The parameters that can be controlled during injection molding are pre-injection chamber and mold temperatures, injection and holding pressures, and injection and holding times. Typically, in biopolymeric systems, the temperature of the pre-injection chamber approximates the glass transition temperature of the blend. This temperature is chosen so that the viscosity of the dough-like blend is minimal, ensuring the complete filling of the mold (Perez-Puyana et al. 2016). The viscosity that is achieved with the heating of the blend defines the injection pressure and time since the speed at which the blend passes from the pre-injection chamber to the mold depends on these. As for the mold temperature, it is generally higher than the temperature of the pre-injection chamber in these systems. This temperature allows the crosslinking of the biopolymeric chains, thus generating the bioplastic matrix. Its temperature depends on the desired characteristics in the bioplastic matrix. In this way, if the target is to obtain good water absorption capacity, the temperature used will be the lowest allowed so that the bioplastic matrix has mechanical properties that prevent it from disintegrating when absorbing water, but without losing the pores of this structure that allow collecting water (Fernández-Espada et al. 2016a). On the other hand, if the mechanical properties are to be prioritized, the chosen mold temperature will be the maximum allowed before the disintegration of the biopolymer matrix. In this case, the morphology of the bioplastic matrix will have smaller pores, but the structuring achieved will improve the final properties of the system.

Finally, the holding pressure and time do not usually cause significant changes in bioplastics, but they can reduce or increase the cure speed of the bioplastic, making the cure time longer or shorter.

16.3.2.4 Additional Processing Treatments

As was previously mentioned, the manufacture of bioplastic matrices involves the use of at least two components (the biopolymer and the plasticizer). Therefore, it is essential to obtain a homogeneous mixture of raw materials to achieve a bioplastic matrix that is as regular as possible (Fernández-Espada et al. 2016b; Carvajal-Piñero et al. 2019). In extrusion processing, this mixing is done by the action of the endless screw. However, in the other two processes (compression and injection molding), a prior mixing stage is required to obtain a well-homogenized blend. At this stage, the ratio of raw materials used is very important. In this sense, if a very high plasticizer ratio is used, the blend will be very fluid, which may make it difficult to handle during processing, and the plasticizer excess will be typically exuded from the bioplastic matrix. On the contrary, if the plasticizer ratio is very low, the blend will be sandy, presenting great resistance to flow and tending to form defects due to the difficulty to fill the cavities of the mold (Fernández-Espada et al. 2016b). In the case that additives are incorporated, the added percentage of these must also be controlled. Thus, it has been found that increasing the percentage of additives generally improves the properties of the bioplastic matrices in the first place. However, the percentage reaches a maximum from which these properties worsen due to the fact that the interaction between the polymer chains may be impaired by the additive (Gamero et al. 2019). Another factor to consider in this mixing stage is the mixing temperature. Most systems can be homogenized at room temperature, using adiabatic conditions, although there are some systems that need to increase their temperature to reach an optimal homogenization level. In these cases, it is necessary to study the thermal transitions of the blends, since excessive heating can generate premature plasticization, which makes it impossible to be processed, even leading to the degradation of the biopolymeric system (Félix et al. 2016).

There are also other additional treatments performed on bioplastic matrices to improve their final properties. In this context, some authors use the functionalization of the polymeric matrix to improve the water uptake capacity of the materials (Cuadri et al. 2018; Capezza et al. 2019). This functionalization consists in increasing the water-collecting groups present in the biopolymers. One of the most common chemical modifications is the acylation of amino acid residues with acid anhydrides, increasing the number of carboxylate anions (COO^-) present in the bioplastic matrix. These groups are large collectors of water, increasing the hydrophilic character of bioplastic matrices. Another modification made in this context is to increase the pH of the biopolymer above its isoelectric point. In this way, inducing a basic medium in biopolymers causes a greater number of negative charges in their chains, which can generate hydrogen bonds with water molecules (Álvarez-Castillo et al. 2020b).

Among the mechanical properties, physical, enzymatic, and chemical modifications can be made in order to improve the mechanical resistance of bioplastic matrices so they can compete with conventional plastic. However, enzymatic and chemical changes are usually avoided in bioplastic matrices used for agriculture and horticulture, since they are in contact with food products and can contaminate these with toxic substances. Among the physical strengthening processes to which these materials can be subjected, heat treatment up to a temperature around 50 °C (also known as dehydrothermal, DHT) is the most studied. This treatment is carried out at a moderate temperature, with the aim of facilitating the movement of the biopolymeric chains to bind each other, thus preventing the denaturation or degradation of the bioplastic matrices (Jiménez-Rosado et al. 2020d). Lately, other techniques are being tested to achieve the strengthening of the bioplastic matrix in a shorter time than in the dehydrothermal treatment. In this sense, great advances have been found with ultrasound and microwave treatments (Karki 2009; Ghasri et al. 2019). These techniques have reduced the processing time from several hours to a few seconds. At the industrial level, this reduction confers an increase in the production of bioplastic matrices by reducing the manufacturing time. However, the implementation of these treatments requires higher initial assets than in the case of the DHT stage, which is why many researchers are focused on mitigating the time/costs ratio to optimize the process (Jiménez-Rosado et al. 2020d).

On the other hand, bioplastic matrices need posttreatments to remove the plasticizer if they are used for the controlled release of nutrients or pesticides (Jiménez-Rosado et al. 2020c). In general, the plasticizers used in these bioplastic matrices are highly hydrophilic; thus, they are easily removed with water. In agriculture and horticulture, a lot of water is used to irrigate crops, so this water generates an uncontrolled release of plasticizer. The substances used as plasticizers in bioplastic matrices for agriculture and horticulture are not harmful, so their release does not generate any toxicity or contamination problems. However, when the bioplastic matrix holds nutrients or pesticides, they are unwantedly released with the plasticizer, making the release faster than expected. To prevent this effect, the plasticizer is removed before use, leaving a bioplastic matrix with additives but without plasticizer. An easy technique to remove the plasticizer once the bioplastic matrix has been formed entails an immersion stage in water since it is highly hydrophilic. However, this process generates two problems. Firstly, the nutrients and pesticides used incorporated into bioplastic matrices are also highly hydrophilic. Thereby, at this stage, there is a loss of more than 80% of these additives, reducing the efficiency of the process and making the final product more expensive. To solve this problem, different studies are testing alternative methods for the inclusion of the additives, such as incorporating them in the same step of removal of the plasticizer instead of in the initial blend. However, these processes involve greater costs, which do not make them economically viable. Another route of study is the use of other immersion solvents, where the plasticizer is soluble, but the additives are not, such as ethanol. This path is the one that seems to have the highest potential, although the products generated are still under development. The second problem comes from the need to dry the bioplastic matrix once the plasticizer has been removed. Although a drying step seems simple, removal of the plasticizer causes the bioplastic matrices to stiffen upon drying, and in many cases, they lose their shape.

16.4 Characterization of the Properties of Bioplastic Matrices

Once the bioplastic matrices are manufactured, they must be characterized. These measures allow evaluating the properties of each of the bioplastic matrices produced, allowing to catalog the characteristics conferred according to the raw materials used and the processing method carried out (Fernández-Espada et al. 2016a). Furthermore, this characterization allows evaluating the reproducibility of bioplastic matrices, avoiding irregularities and lack of quality at an industrial level. Generally, there are norms that standardize the methods carried out for the characterization of materials in each of the industrial sectors. However, the relative novelty of the bioplastic matrices in agriculture and horticulture has no designated norms for them till date. Normally, due to the similarity of bioplastic matrices with conventional plastics, standards are adapted for the evaluation of bioplastic matrices. In this way, the same tests are used, but smoothing the measurement conditions (Razza et al. 2020).

16.4.1 Microstructural and Mechanical Properties

First, the mechanical and microstructural properties can be evaluated. These properties are directly related to each other and to the functional properties of bioplastic matrices. In general, bioplastic matrices have a regular porous microstructure throughout their surface, where the walls are formed due to the crosslinking of the biopolymer chains (Cuadri et al. 2016). Pore sizes depend on the degree of crosslinking produced. Thus, if the processing conditions generate low crosslinking, the bioplastic matrices will have a greater free volume, increasing at higher degrees of crosslinking (Liu et al. 2005). This change in structure causes bioplastic matrices to have different mechanical properties. Thus, the more porous bioplastic matrices possess the worse mechanical properties. Thus mechanical properties ratio must be taken into account while processing the bioplastic matrices in order to obtain the best pore size. In this sense, when bioplastic matrices are used in applications such as seedbeds or plant guides, the mechanical properties of the pores will be prioritized, since in this case it is not necessary to have pores in the structure. Conversely, in water absorption or substance release applications, it is important to have many pores to host water, fertilizers, and pesticides. Furthermore, the fact that these pores are distributed throughout the matrix means that there can be a controlled release during biodegradation. The microstructure of bioplastic matrices can be evaluated using microscopic techniques. In this context, scanning electron microscopy (SEM or Cryo-SEM) is usually performed to analyze the structure of bioplastic matrices. This technique allows observing micro and nanometric sizes, making it possible to evaluate the surface pores (Jiménez-Rosado et al. 2020a). To carry out these analyses, the samples must be covered with a thin layer of conductive material, since the insulating character of the bioplastic matrices decreases the quality of the micrographs. Furthermore, different porosimetry techniques have been used to evaluate the general porosity of the matrices. Among them, mercury intrusion porosimetry is one of the most used, and it allows obtaining the pore size distribution throughout the bioplastic matrix.

In addition to the porous structure and porous distribution, it is also necessary to evaluate the presence of additives when they are incorporated into the bioplastic matrices. For this, energy dispersive spectroscopy (EDAX) or environmental scanning electron microscopy (ESEM) is used, which makes it possible to obtain the distribution of the additives throughout the bioplastic matrices. This helps to know how the different additives are released since the closer they are to the surface, the earlier they are released (Bourny et al. 2017; Jiménez-Rosado et al. 2020c). As for the mechanical properties, these can be determined by static and dynamic tests. Static tests are used to determine the maximum mechanical capacities of bioplastic matrices, while dynamic measurements are used to assess their stability over time (Sadasivuni et al. 2020). The stresses most commonly measured in this type of bioplastic matrices are tensile, flexural, and compressional stresses, since they are the stresses that they are subjected by the crops and the soil. Modifications to the ISO 547, ISO 604, and ISO 178 standards are used to carry out these tests, where test parameters were smoothed, such as the strain speed or the range of frequencies and strains studied.

16.4.2 Biodegradation

Biodegradability is the most important characteristic that defines this type of material. Thanks to it, there is an adjusted half-life for the application to be developed, preventing the accumulation of plastics in cultivated soil. The biodegradability of a polymer basically depends on its polymer structure and is independent of its nature. The consideration of a polymer as biodegradable is regulated by the EN 13432, ASTM D6400, and ISO 17088 standards, indicating the necessary tests to be carried out so that the polymers are considered biodegradable (Ruggero et al. 2019). These tests are usually carried out by introducing the bioplastic matrix together with a compost made from an inert substrate in a closed container. This container is introduced into a stove where the temperature can be controlled, measuring the amount of CO_2 released over time. The temperature at which the test is carried out depends on the final application of the bioplastic matrix. Thus, for applications in agriculture and horticulture, ambient temperatures are usually used, varying between 10 and 40 °C. In addition to CO_2 measurements, matrix degradation can also be assessed visually or by weighing them. The optimal biodegradability is estimated according to the desired duration of the bioplastic matrix. As harvesting time of some crops are 1 month (e.g., lettuce, pepper, zucchini), others may take up to a year (e.g., potato or carrot). Thus, depending on this duration, it will be optimal for bioplastic matrices to have one biodegradability or another. This change in biodegradability can be efficiently optimized in bioplastic matrices during processing. In this way, when a longer biodegradation time is required, the bioplastic matrices undergo treatments that generate a greater crosslinking of the biopolymer chains, doing the opposite when shorter biodegradation times are required (Bezirhan Arikan and Bilgen 2019). This modulation of biodegradability is driving the market for other biodegradable materials such as paper.

16.4.3 Water Uptake Capacity

The water absorption capacity is an essential property of bioplastic matrices made to supply water. Water absorption capacity is the ability to retain water without losing the structure of the bioplastic matrix (Fernández-Espada et al. 2016a). This capacity allows bioplastic matrices to be used as a source of water, supplying it during its biodegradation. ASTM D570 is used to measure this capacity. In this way, a rectangular bioplastic matrix (70 × 10 1 mm^3) is introduced into a closed vessel with 300 mL of distilled water for 24 h. Thus, the water uptake capacity is calculated using Eq. (16.1).

$$\text{Water uptake capacity}\ (\%) = \frac{m_3 - m_2}{m_2} \cdot 100 \tag{16.1}$$

where m_2 is the weight of the bioplastic matrix after immersion in water and m_3 is the weight of the bioplastic matrix after drying the water that it has absorbed. This drying can be carried out by thermal treatment or lyophilization, but it must be taken into account that the matrix does not degrade after this treatment so that the measurement is as reliable as possible. In addition to these measurements, the soluble loss capacity can be evaluated using Eq. (16.2). This value allows knowing how much matrix material is lost during water absorption, which estimates its stability during absorption.

$$\text{Soluble matter loss}(\%) = \frac{m_3 - m_1}{m_1} \cdot 100 \tag{16.2}$$

where m_1 is the weight of the bioplastic matrix before the immersion in water.

It is worth mentioning that, although the standard estimates are measured with distilled water, but practically for application in agriculture and horticulture, it is important to carry out this test with running water. This is because the crop is irrigated with this water, which contains salts and can modify the absorption capacity of the matrices. In fact, tests carried out with running water give lower water absorption results due to the presence of salts in them. These salts generate electrostatic repulsions that do not allow the bioplastic matrices to swell in order to retain large amounts of water (Guilherme et al. 2015).

16.4.4 Controlled Release of Fertilizers

There are fewer studies focused on the characterization of this property. This means that there is no specific regulation on how to carry out these tests. However, in the few works conducted in this area, two trends are always followed:

- Study of fertilizer release in water (Cong et al. 2010; Essawy et al. 2016): this test consists in immersing the bioplastic matrix with incorporated fertilizers in 300 mL of running water. Since the incorporated fertilizers are salts, they produce a change in the conductivity of the medium when they are released into the water, which is related to the amount of fertilizer released. In addition, if this study is followed over time, an estimation of the release time can be accomplished, which will correspond to the time required for the conductivity of the medium to remain unchanged for more than 1 h. The data obtained with these tests can be correlated with the release that occurs in the soil, which allows estimating the kinetics of fertilizer release.
- Study of fertilizer release in farmland (Hanafi et al. 2002; González et al. 2015; Essawy et al. 2016): in this case, the bioplastic matrices are buried in the soil using a device like the one observed in Fig. 16.8. In this way, a constant amount of water is introduced (normally 20 mL) above the device once per day, in order to simulate regular irrigation, collecting the leachate obtained from the bottom.

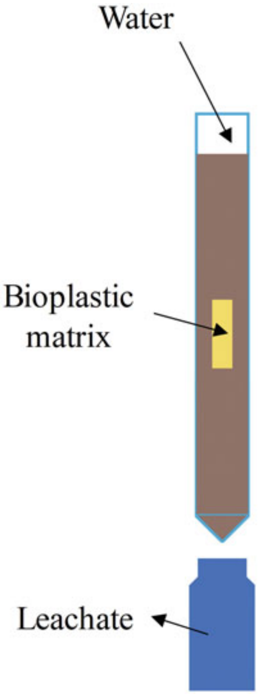

Fig. 16.8 Equipment for the study of fertilizer release in farmland

> This leachate is evaluated by conductivity, and variations in conductivity can be correlated with the generated release of fertilizers. In this way, the behavior of the bioplastic matrices in the farmland can be evaluated more precisely, obtaining a better estimation of the release kinetics. However, this test is longer and more expensive than the water test, so it is only used when the processing of the bioplastic matrix is already optimized, obtaining only the values of the most optimal systems.

These studies can be completed by checking how much fertilizer remains in the matrix after a test time. This is achieved by evaluating the matrix using spectroscopic methods where the presence of elements up to trace levels can be determined. The most widely used is inductively coupled plasma spectroscopy (ICP-AES), where all the nutrients that can be incorporated into the crops can be analyzed together in the same analysis, which makes the characterization faster (Jiménez-Rosado et al. 2020a).

16.4.5 Plant Analyses

All the mentioned tests are used to previously evaluate the properties of bioplastic matrices and, thus, to be able to choose the one that presents the best properties according to its final application. However, before bioplastic matrices can be commercialized, they need to be tested in real crops to ensure the evaluated properties. For this, it is necessary to choose a suitable crop with a cultivation time similar to the biodegradation of the studied bioplastic matrix, evaluating the selected system with a positive crop (comparing the bioplastic matrix with an efficient commercial plastic system) and a negative crop (without using any system). The effectiveness of the studied bioplastic is checked depending on its application. If the application is to serve as a seedbed or plant guide, it will be checked if adequate biodegradation of the bioplastic matrix has taken place, without generating toxicity to the crop and growing adequately. On the other hand, if it is for water supply, it is checked how much water is necessary to irrigate each system. In controlled pesticide release systems, their efficiency is verified by using them in crops where the pests are attracted to the devices. Lastly, in order to test the controlled release of fertilizers, the presence of defects due to nutrient deficits is visually evaluated, in addition to the nutritional evaluation of the plant, to see its nutritional improvement. In this context, post-cultivation soil studies can also be performed to examine the possible accumulation of fertilizers in the soil (Martin-Closas et al. 2014).

16.5 Conclusion

To sum up, bioplastic matrices have opened a new field in the agriculture and horticulture sectors, generating a new restructuring of the sectors by increasing the efficiency of crops and reducing the amount of conventional plastics used in them.

Thus, devices with the desired biodegradability can be obtained for different types of crops and several applications, reducing the use of conventional plastics in these sectors. However, bioplastic matrices still need a lot of research before they can really compete with conventional plastics. In this context, the manufacturing process must be optimized. In addition, it is important to create standards that regulate the characterization of bioplastic matrices in order to homogenize their market.

16.6 Future Perspectives

The future trends in horticulture advance from different points of view with a common goal: the optimization of products to improve production. In this sense, several lines of research can be highlighted, such as improving the general process and obtaining a better and more specific characterization of the products. In general, horticulture optimization can be carried out from a manufacturing point of view, as is shown in the study of Pearce et al. (2018) with the use of lean management practices. For example, applying temperature to dry the bioplastic matrices generates a denaturation of the biopolymer, thereby decreasing the functionality of the bioplastic matrices. This aspect could be improved by eliminating water through sublimation, but the need to buy specialized equipment for this generates an economic investment that increases the economic return time of the investment industries in these materials. This has led to further research in this regard, looking for systems that do not require the incorporation of the plasticizer to create the bioplastic matrix. However, these systems are still in a very experimental phase; thus, a lot of research is required before they can join the industry. Further research can focus on the development of fertilizers and their influence on the crop productivity, as reported in the recent studies by Prabakaran et al. (2018) and Ostadi et al. (2020). In this way, a new field of research opens up in which not only do we have to pay attention to fertilizers, but also to their release control, as was described by Cole et al. (2016) on the effect of an individual application or controlled combinatory release of nutrients (nitrogen, phosphorus, calcium, and magnesium).

As has been seen in previous sections, in addition to the processing of the materials, adequate characterization of them is convenient to confirm that the products have the necessary properties for the application in which they are intended to work. The aforementioned techniques are the most studied in this field of research, which still has innovation in this regard, such as the studies by Nturambirwe and Opara (2020) on the development of defect detection methods using non-destructive techniques for horticultural products (Nturambirwe and Opara 2020). In this sense, it is worth highlighting the study by Qin et al. (2019) about the promotion of Raman spectroscopy and imaging techniques for the inspection of the quality and safety of horticultural materials. On the other hand, the application of emerging technologies is a field of research that has been booming in recent years. In this sense, the application of nanotechnology plays a crucial role. However, the obtained products have not only advantages but also drawbacks that must be taken into account in order to remedy them. In this sense, the review by Feregrino-Perez et al. (2018) on the strengths and aspects to be improved about the impact of nanotechnology in horticulture highlighted that efforts must be applied for the creation of intelligent fertilizers (delivery of fertilizers, bio-fertilizers, micronutrient supply, nanoherbicides) aiming for improving plant disease resistance, growth, and nutrition.

Regarding product development, there are more and more studies aimed at developing bioplastic matrices from food waste valorization (Jiménez-Rosado et al. 2018; Tsang et al. 2019). A relevant aspect that could boost the use of bioplastics in horticulture is to develop a circular economy in this field (Burgos et al. 2016), as De Corato proposes in his analysis of waste recycling in the agricultural and horticultural industry (De Corato 2020). A similar, although a more general study was carried out by Rybicka et al. (2016) on the real viability of using bioplastic recycling techniques for industrial application due to legislative restrictions, which are increasingly demanding regarding the environmental impact of waste. In this sense, a possible environmental evaluation of the product/process could be explained by means of a description of a life cycle analysis (LCA), highlighting the study by Udugama et al. (2017), who proposed a preliminary analysis of the implementation of the Resource Recovery from Bio-Based Production Processes. Furthermore, as Michael defined in 2017, “with the development of bioplastics, the industry is closer to achieving sustainability via reducing the carbon footprint and reliance on fossil fuels.”

The relevance that the field of horticulture is taking is reaching levels that were unimaginable some years ago; for example, Vermeulen et al. (2019) suggested the development of a program to boost the innovation and development of the circular greenhouse by adopting concepts and technologies from space since it is inherently focused on circularity because of scarce resources. In the study, it becomes clear that not only terrestrial horticulture could benefit from space technologies, but that human space exploration could equally benefit from the technical and tacit knowledge of horticulturists for food production in space (Vermeulen et al. 2019). In essence, the search for new materials and the use of existing ones to develop new products for the horticultural industry is continuous, so we are in an area of knowledge with great potential that can lead to systems with surprising properties that we cannot imagine today.

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Altering the Hydrophobic/Hydrophilic Nature of Bioplastic Surfaces for Biomedical Applications

17

Zulfiqar Ali Raza, Rizwana Khatoon, and Ibrahim M. Banat

Abstract

Biologically produced polymers and that ex situ synthesized from biogenic precursors have captured the attention of researchers and industrialists due to their diversity, biodegradability, biocompatibility, and renewability. They find diverse applications in enormous fields of science and technology including packaging, foods, textiles, water treatment, cosmetics, sensing, and microelectronics. The chemistry of surfaces is a window for originating new properties and opportunities. Based on their surface affinity, bioplastics may be either hydrophobic or hydrophilic which directs their potential applications. Their surface chemistry may be tailored by imparting desirable functionalities for improving their physicomechanical properties. This can be achieved through different physical, chemical, and biological approaches. Such bioplastics with improved hydrophilicity are desirable in the fields of medical science, nanotechnology, sensing, and so forth. The objective of the present chapter is to present recent developments in tuning the surface properties of bioplastics using different approaches for biomedical applications.

Keywords

Biomedical · Bioplastic · Biopolyester · Hydrophilic · Hydrophobic · Polymer · Surface modification

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17.1 Biopolymers

Polymers formed in biological systems (like animals, plants, or microorganisms) through complex metabolic processes or similar others produced through synthetic biochemistry from biological precursors (like, sugars, fats, vegetable oils, proteins, amino acids, resins, etc.) are usually termed as natural or bio-polymers (Mohan et al. 2016). Polysaccharides such as starch, cellulose, and chitin; proteins like enzymes and keratin; and bio-polyesters like poly(hydroxy alkanoate) (PHA) especially poly (3-hydroxybutyrate) [P(3HB) or simply PHB] are all counted in biopolymers. Those biopolymers with sustainable mechanical strength and other polymeric properties are often required for diverse applications. They are non-toxic and ultimately biodegraded to CO_2, H_2O, and some secondary metabolites (Mohiuddin et al. 2017). Such biopolymers have vast applications in agriculture, packaging, environment, medical, and nanotechnology-related industries mainly due to their biocompatible, biodegradable, and nontoxic nature (Pattanashetti et al. 2017).

In recent years, attention has been directed toward biopolymers because they are produced from renewable sources compared to synthetic plastics obtained from depleting crude oil sources. Also, the disposal of these synthetic plastics is challenging as their degradation in landfills is very slow, incineration is costly and generates polluting gases, and recycling is a lengthy process which changes their inherent properties too. The replacement of these traditional plastics, therefore, with naturally occurring biopolymers such as polysaccharides, polylactides, and aliphatic bio-polyesters among many others has been recommended due to environmental concerns (Jennyangel and Dhandapani 2013). One criterion for the above purpose, however, is that these biopolymers should have properties comparable to petroleum-based polymers.

During the past few decades, synthetic polymers had been used as packaging materials as they are soft, light in weight, flexible, and transparent. The non-degradability of these polymers creates ecological issues so eco-friendly packaging materials (biodegradable) have been demanded (Rhim and Ng 2007). Advanced biopolymers such as proteins, cellulose, starch, and poly(lactic acid) (PLA) have extensively been used as packaging materials for food and pharmaceutical products (Álvarez et al. 2017). On the other hand, rock oil-based polymers are not biodegradable, and among them, some are challenging to be reprocessed or recycled as they have complex molecular structures resistance to physical, chemical, and biological degradations (Maitz 2015).

Recently, a lot of work has been carried out in the development of bioplastics to achieve desirable features. Such products find applications in disposable cutlery and plates, garbage bags, shipping, and food packaging materials (Balart et al. 2018). The biopolymer compounds perform various sets of functions at their inherent cellular locations. For instance, polysaccharides play a key role in cells for intracellular communication, as cell wall structures, capsular layers, or defensive barriers around the cells and as adhesives (Jennyangel and Dhandapani 2013). Likewise, proteins have a role as structural units, catalysts, cell functions controller and adhesives, and lipids serve for energy storage and barriers in cell membrane structures. Thereby, the inherent functions of biomaterials may find their in vitro applications in various fields (Mkandawire and Aryee 2018). For instance, lipids with different degrees of saturation and lengths of fatty acids are used as surfactants and emulsifiers in cosmetics (Duprat-De-Paule et al. 2018). Biopolymers like silk, chitosan, and PLA are used as implant devices in supporting structures for bones, heart, ears, eyes, hips, and cardiovascular valves (Rebelo et al. 2017). Various types of biopolymers like casein, chitosan, zein, and wheat gluten are used in paper coatings with better barrier properties against moisture, oxygen, nitrogen, and CO_2 (Khalil et al. 2018). They have also been used as anti-desertification agents to inhibit soil erosion through better inter-particle cohesions. The biopolymers like β-glucan and xanthan gum are used to improve moisture retention in the soil through hydrogel formation that improves the plants' growth (Chang et al. 2015).

The applications of biopolymers in the medical field could broadly be divided into three classes: drug delivery systems, healing products for wound care, and surgical implants and devices. The devices made up of biodegradable polymers might be used as implants in the human body without any requirement of post-surgery procedures to remove them after healing (Desmet et al. 2009). Biopolymers in the form of nanostructures had been used in remedial applications particularly as drug delivery vehicles and tissue engineering scaffolds (Saska et al. 2018). Biopolymers are also becoming attractive in the development of vapor and gas sensors (Krebsz et al. 2017). Electrical noses have been developed using specific biocomposites that find vast applications in environmental monitoring, disease control, industry, and public security (Vieira et al. 2011). Such applications could further be improved by developing the biopolymer composites in the form of novel nano-bio materials (Raza et al. 2020). Besides this, recently, flexible, lightweight, and economical photodetectors based on biocomposites have also been demanded (Mohiuddin et al. 2017).

17.2 Classification of Biopolymers

Based on production mode and sources, biopolymers can be categorized as follows:

(a) Polymers derived from biomass including polysaccharides (like starch, cellulose, lignocellulosic products, chitin, and its derivative and pectins), proteins (from plants like zein, gluten, soya, and from animals like casein, gelatin, whey), and lipids (like castor oil, animal fats)
(b) Polymers produced by microbes (like PHA, PHB, and poly(hydroxybutyrate co-hydroxyvalerate (PHBHv))
(c) Chemically prepared polymers from monomers of biological origin like PLA.
(d) Chemically synthesized polymers from diverse sources include poly (caprolactone) (PCL), polyesteramides (PEA), and aliphatic and aromatic co-polyesters (Vieira et al. 2011)

A layout of the present study expressing biopolymers' types and major surface modification approaches is shown in Fig. 17.1.

17.2.1 Hydrophilic Polymers

Historically, materials comprising of molecular and ionic species had been termed as hydrophilic if they were water-soluble, while hydrophobic materials being insoluble in aqueous systems. The hydrophilic surfaces absorb water from the surrounding environment being hygroscopic, e.g., sugar and sodium chloride (Israelachvili 2011). Some naturally occurring inorganic salts and synthetic and natural organic polymers (having polar functionalities) are also water-loving. So, by simple solubility test, one could identify the hydrophilicity of various substances. The hydrophilic substances are so because of the prevalence of polar groups on their surfaces. Such surfaces follow the rule of "polar spreads on polar" or "like spreads on like" (Drelich et al. 2011). However, the concepts of surface chemistry have now been evolved, so the hydrophilicity or wettability is measured based on the water drop contact angle on a test surface. This shows the extent of spreading of water drop on a solid surface when a physicochemical interaction between the solid and the liquid drop is established. The surfaces having a contact angle lesser than 90° are termed as hydrophilic due to polar grouping being developed with polar groups of water molecules resulting in water drop spreading; thus, lowering the resultant contact angle. The introduction of hetero-atoms other than fluorine in the hydrocarbon structure, or the introduction of double or triple bonds in hydrocarbon molecule, or the presence of charged groups such as NH_2, –OH, or –COOH on a surface enhances its polarity as well as surface hydrophilicity. The improved hydrophilicity promotes protein and cellular attachments with the targeted surfaces (Drelich et al. 2011; Zeng et al. 2017).

17.2.2 Hydrophobic Biopolymers with Their Hydrophilic Potential

As stated above, the surfaces having a contact angle greater than 90° are termed as hydrophobic. The energy associated with the intermolecular forces at the interface of two phases is known as surface energy. Thereby a hydrocarbons' network with zero functionality having minimum surface energy exhibits hydrophobicity. The surfaces having contact angles greater than 150° are called super-hydrophobic, and if it were closer to 0°, then it is termed super-hydrophilic. In the following sections, we present different hydrophobic polymers, their functionalities, and approaches to make them more hydrophilic to explore their potential use in diverse biomedical applications.

17.2.2.1 Chitin

Chitin is a natural poly(saccharide) chemically named as poly(β-(1–4)-*N*-acetyl-D-glucosamine (Fig. 17.2a). It naturally occurs in several organisms being the most abundant biopolymer after cellulose. Chitin is found as a structural constituent of

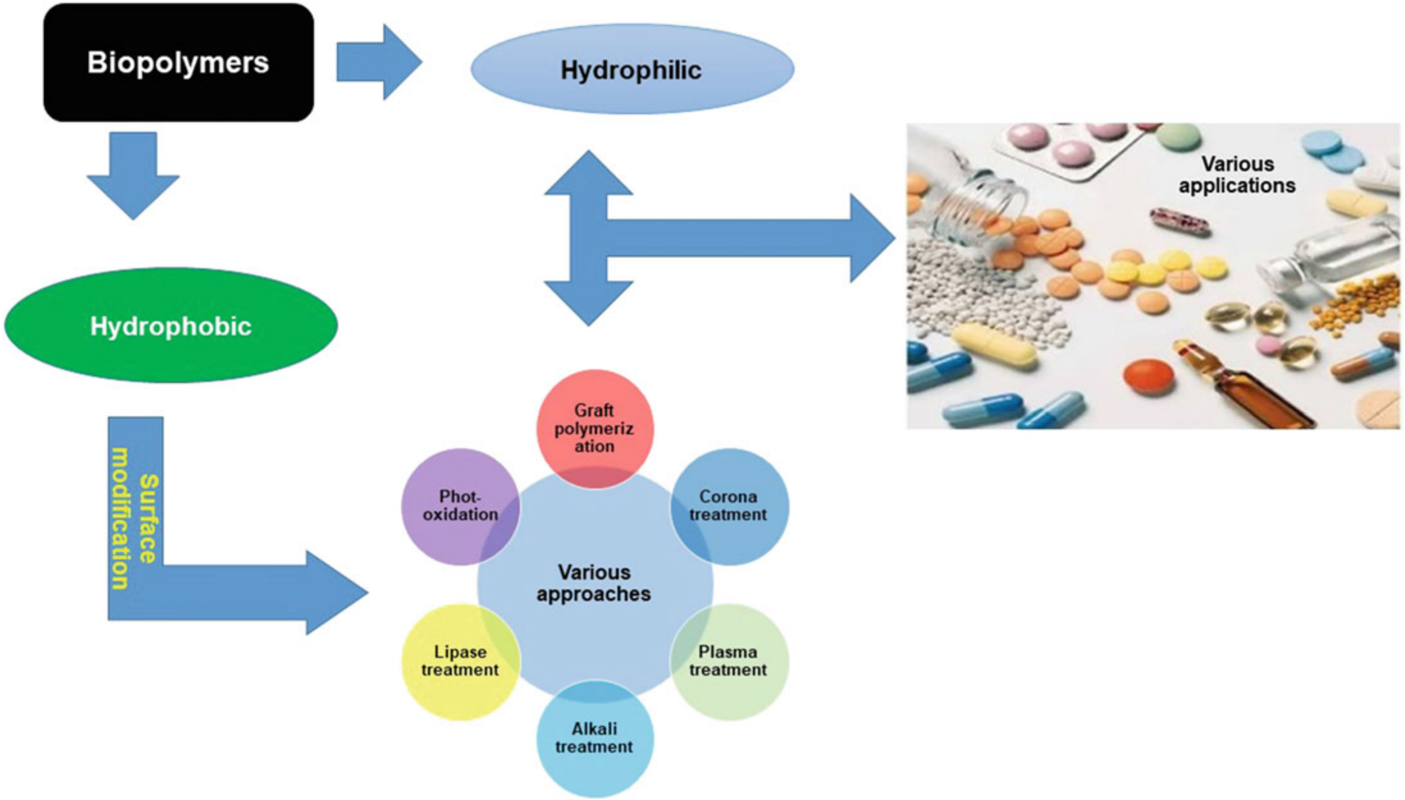

Fig. 17.1 A layout of the present study—biopolymers' types and major surface modification approaches

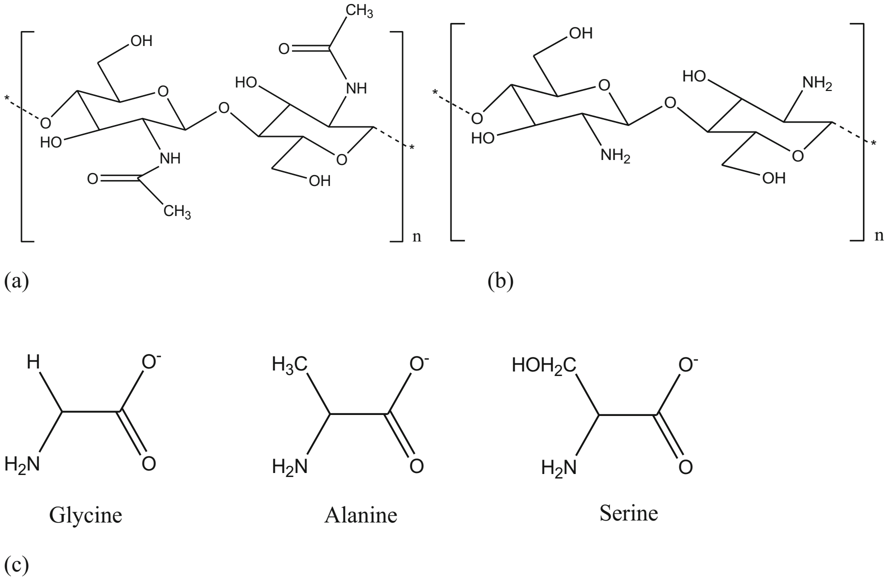

Fig. 17.2 Structures of (**a**) chitin, (**b**) chitosan, and (**c**) chemical structures of some amino acids being congeners of silk fibroin

exoskeletons of several arthropods like crabs and shrimps and is present in cell walls of some yeasts like *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe (*Sánchez-Vallet et al. 2015*)* and fungi like *Aspergillus fumigatus* and *Candida albicans* (Peter 2002). It is also biosynthesized in numerous lower plants (centric diatoms and in *chrysoflagellate* alga *Poterioochromonas*) and lower animals (in the bristles of hydrozoan thecae and polychaetes) (Muzzarelli 2010).

In 1811, Henri Bracon discovered chitin and named it fungine. Odier, in 1823, found a similar material in plants and insects and named it chitin which thereafter was called chitin. It is a white, hard, and inflexible nitrogen-containing poly(saccharide) (Azuma et al. 2015). On the industrial scale, chitin is isolated from crustaceans via acid treatment followed by alkaline extraction. Chitin is a biocompatible, biodegradable, non-toxic, and antimicrobial material so used in the formation of membranes, gels, nanofibers, microparticles, nanoparticles, and scaffolds (Islam et al. 2017). It is water-insoluble due to strong inter- and intra-molecular H-bonding between neighboring –OH and $–NH_2$ functional groups and is hydrophobic due to acetyl amine group on each monomer unit (Zargar et al. 2015).

17.2.2.2 Chitosan

Chitosan is derived by alkaline deacetylation of chitin, although the deacetylation is mostly incomplete. Based on its structural features, chitosan is a copolymer of *N*-acetyl glucosamine and glucosamine units (Fig. 17.2b). It contains three functionalities of hydroxyl, amino, and acetamido groups (Zhang et al. 2010). The main characteristics of chitosan are its degree of deacetylation and molecular mass.

Its molecular mass ranges from less than 50 kDa to about 50 kDa (Soni and Ghosh 2017). Chitosan is water-insoluble under alkaline conditions but becomes soluble dilute acid media of acetic and formic acids due to the presence of an amino group on chitosan. The amino groups are protonated which makes the chitosan water-soluble (Abid et al. 2019). The presence of –OH and –NH_2 groups makes the chitosan hydrophilic which facilitates its chemical modification to achieve new functionalities (Tangpasuthadol et al. 2003) making it suitable in pharmaceutical and tissue engineering applications (Depan and Singh 2015).

The chitosan has good biocompatibility, biodegradability, and non-toxicity so it is efficiently used in pharmaceutical and biomedical fields for targeted drug delivery, wound healing, and implanting purposes (Muxika et al. 2017). Regarding its film-forming characteristics, chitosan finds potential applications in food packaging, e.g., as edible films (Yuan et al. 2016). Besides, chitosan also expresses antifungal, antibacterial, and therapeutic properties suitable for wide applications in agriculture, medical, and other fields (Ouyang et al. 2017).

17.2.2.3 Silk Fibroin

Silks are naturally occurring proteins produced by silkworm *Bombyx mori*, mites, spiders, bees, and so on. The silk obtained from different sources contains different structures, composition, and properties. Silk as a thread had been used as biomedical sutures (Zhao et al. 2015). The silk consists of two basic proteins, viz., making silk fibroin (SF) nearly 75% of the thread core and sericin being 25%, responsible for binding the silk fibroin into fibers. The SF is a fibrous protein with a semi-crystalline structure that makes the silk matrix strong, while sericin, present between SF, is amorphous protein acting like glue or an adhesive binder to maintain the dimensions of silk fiber. The SF is separated from the sericin via degumming (Li et al. 2012). The SF consists of non-repetitive hydrophilic and repetitive hydrophobic polypeptide arrangements that contain heavy (H) and light (L) chains of polypeptides bonded through a disulfide linkage at the terminal C of two chains; as a result, an H-L complex is formed (Koh et al. 2015). Glycoprotein 25 is also bonded to the H-L complex through hydrophobic interactions. The H-chain, L-chain, and glycoprotein 25 are linked in the ratio of 6:6:1 (Zhao et al. 2015). The basic structure of SF protein consists of three amino acids [viz., glycine (Gly), alanine (Ala), and serine (Ser)] (Fig. 17.2c) which are mostly arranged as Ser-Gly-Ala-Gly-Ala-Gly (in a ratio of 3:2:1). The SF chain also consists of amino acids (like valine, tyrosine, aspartic acid, etc.) having large polar groups. The hydrophobic repetitive units make the crystalline region of the SF, whereas the hydrophilic non-repetitive units form the amorphous region of it (Koh et al. 2015).

The H-chain contains 12 repetitive hydrophobic domains (composed mainly of glycine, alanine, serine and threonine, valine, and tyrosine in small amounts) which are linked through inter- or intra-molecular forces to form the crystalline part of the SF. The primary structure of the H-chain consists of repeating units of GAGAGS, GAGAGY, and GAGAGVGY which are hydrophobic. The hydrophilic non-repetitive domains (which are 11 in numbers) consist mainly of charged amino acids (like lysine, arginine, aspartic acid, and glutamic acid) forming the amorphous part of the SF. The L-chain is elastic and hydrophilic due to the non-repetitive arrangement of the amino acids (Koh et al. 2015). As mentioned above, the compact structure of SF consists of both crystalline and amorphous regions. Silk I (naturally occurring as fibroin) and silk II (regenerated fibroins) are both crystallines in nature. However, due to its highly crystalline geometry and oriented structure, the silk II fibers exhibit reasonably high hydrophobicity as well as mechanical strength (Li et al. 2012). Recently, great interest has been developed using the SF in biomedical applications and biotechnology as such matrixes are sporting in osteoblast and fibroblast cells and in tissue engineering applications (Kim et al. 2014; Koh et al. 2015).

The SF fibers, also, have negligible water holding capacity due to their extraordinary surface smoothness. So, the pigments printed patterns on silk results in poor color production and bleed easily. However, oxygen plasma treatment of SF makes its surface smooth and introduces polar groups such as –OH, –CO, –NH_2, and –COOH on the surface of SF to improve its hydrophilicity (Li et al. 2012). Likewise, UV irradiation has also been applied to make SF fiber (SFF) surface hydrophilic and adhesive with minor changes in crystallinity, density, and strength. For general purposes like dyeing, the improvement in roughness and hydrophilicity of SF fiber increases the uptake of dye and helps fixation of dye molecules. The SFF with improved hydrophilicity is also desirable for cell adhesion (Li et al. 2012; Kim et al. 2014).

17.2.2.4 Poly(Caprolactone)

PCL, an aliphatic polyester, consists of hexanoate repeated units. The PCLs are synthesized either through ionic ring-opening polymerization of caprolactone (Santoro and Perale 2012) or free-radical ring-opening polymerization of 2-methylene1-3-dioxepane (Hajiali et al. 2018). The synthesis of PLC via both routes is shown in Fig. 17.3. PCLs are highly hydrophobic due to the presence of ester linkage in their molecular chains. At room temperature, PCLs are highly soluble in carbon tetrachloride, chloroform, benzene, dichloromethane, toluene, 2-nitropropane, and cyclohexanone while partially soluble in ethyl acetate, acetone, 2-butanone, acetonitrile, and dimethylformamide and insoluble in alcohols, diethyl ether, petroleum ether, and water (Sinha et al. 2004). The mechanical strength, biodegradability, and biocompatibility have made them a good candidate in the biomedical field, e.g., drug

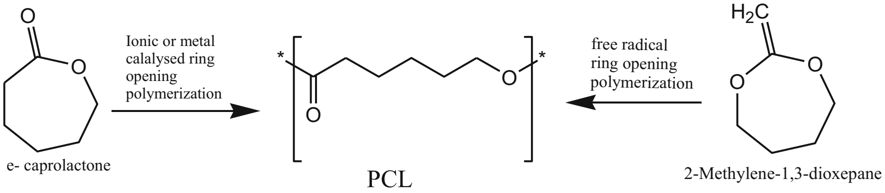

Fig. 17.3 Synthesis of PCL

delivery systems (DDS), durable implants (slowly degrade), and as scaffolds in tissue engineering (McKeen 2012). Due to their hydrophobic nature, PCLs are resistant to strain therefore exhibit a self-cleaning effect (Hajiali et al. 2018).

The surface hydrophilicity of PCLs can be improved by imparting functionality via various surface treatments such as plasma treatment, ozonolysis, photo-induced grafting, and surface oxidation for biomedical applications (Mirhosseini et al. 2016). For instance, argon plasma treatment of 3D electrospun PCL/chitosan/PCL layer-by-layer hybrid scaffold had been carried out for enhancing its cell adhesion and propagation properties. The characterization of treated samples showed a decrease in contact angle (i.e., increment in hydrophilicity) on introducing some nitrogen and oxygen-containing functionalities on the polymer surface (Surucu et al. 2016). The surface of the PCL scaffold could be made hydrophilic by adding pristine graphene via alkaline treatment which might result in improvements to cell attachment and biological activity (Wang et al. 2016).

17.2.2.5 Shellac

Shellac is a naturally occurring polymeric complex resin secreted by lac insect—*Kerria lacca* residing on some plants in India China and Thailand. It contains soft and hard resins, waxes, coloring substances, debris, and moisture (Singh et al. 1969). Overall, shellac is resinous and its components are associated with each other through hydrogen bonding (Obradovic et al. 2017). After dewaxing, it becomes soft between 65 and 70 °C and melts in the range of 75–80 °C. Its average molecular mass is reported as 1006 amu, specific gravity as 1.14–1.21, and acid value being 65–75 (Gardner and Whitmore 1929). It is soluble in alkalies, alcohols, ketones, and organic acids but insoluble in water, esters, glycerol, and hydrocarbon solvents (Wei et al. 2015). The main building units of shellac are aleuritic acid and jalaric acid as shown in Fig. 17.4, which are linked via ester and lactide linkages. So it confirms the presence of carboxyl, hydroxyl, and carbonyl groups in the shellac (Sharma et al. 1983).

Shellac is a renewable, biodegradable, and hydrophobic polymer. It shows thermoplasticity, non-toxicity, and moisture barring features (Limmatvapirat et al. 2004). It has excellent water resistance and is frequently used in glazes on foods like candies and fruit (Wei et al. 2015). It has outstanding film-forming and protective characteristics that make it suitable for paints, food packaging, and drug encapsulation, glossing, sealing, and interior coatings. Due to its weak mechanical properties, it had rarely been used in the medical field, so thermoresponsive biologically active nano-fiber mats (resembling extracellular matrix) had been designed to enhance its usage in wound therapy (Vannuruswamy et al. 2015). The blending of shellac with zein in different ratios had been carried out to formulate shellac-based tablets to be used as carriers for controlled drug delivery (Chongcherdsak et al. 2014).

17.2.2.6 Poly(Lactic Acid)

Poly(lactic acid) is composed of lactic acid (a chiral molecule that exists in two enantiomeric forms, i.e., D and L-lactic acids) and a lactide. In 1932, Carother was first to prepare a low molecular mass ($M_W = 1000–5000$ amu) PLA by dehydrating

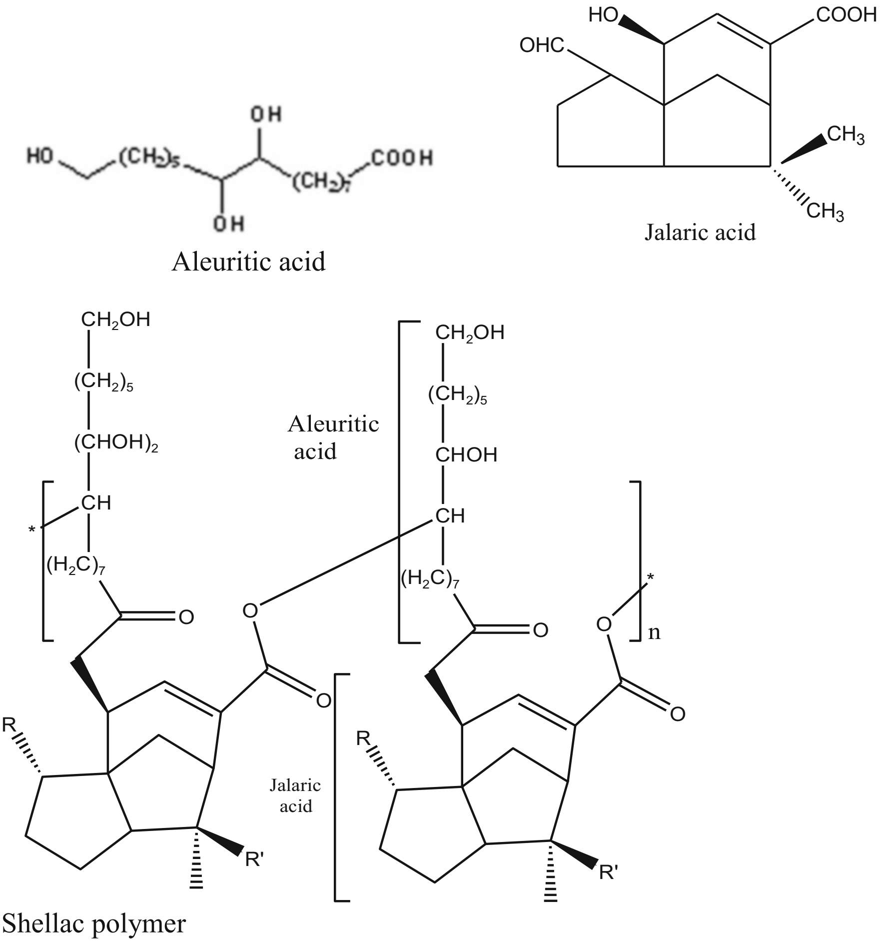

Fig. 17.4 Chemical structures of aleuritic and jalaric acids and the shellac polymer

lactic acid under vacuum. It is either biosynthesized via bacterial (*Lactobacilli species*, e.g., *L. rhamnosus*, *L. delbrueckii*, *L. amylophilus*, *L. bavaricus*, and *L. casei*) fermentation on carbohydrates (e.g., corn starch, wheat, and rice) as carbon sources or synthesized chemically from petrochemical sources via polymerization routes (Jamshidian et al. 2010). Chemically, the production PLA can be achieved through two pathways, viz., ring-opening polymerization and polycondensation (Fig. 17.5). The former gives high molecular mass ($M_W > 100$–1000 amu), whereas the latter gives low molecular mass PLA (MM = 1000–5000 amu) (Jennyangel and Dhandapani 2013). During polycondensation, hydroxyl and carboxyl groups are bonded and water is eliminated (Rahmayetty et al. 2017). During the ring-opening polymerization, first, lactic acid undergoes polymerization to give PLA oligomers of low molecular mass, which may further undergo catalytic depolymerization to

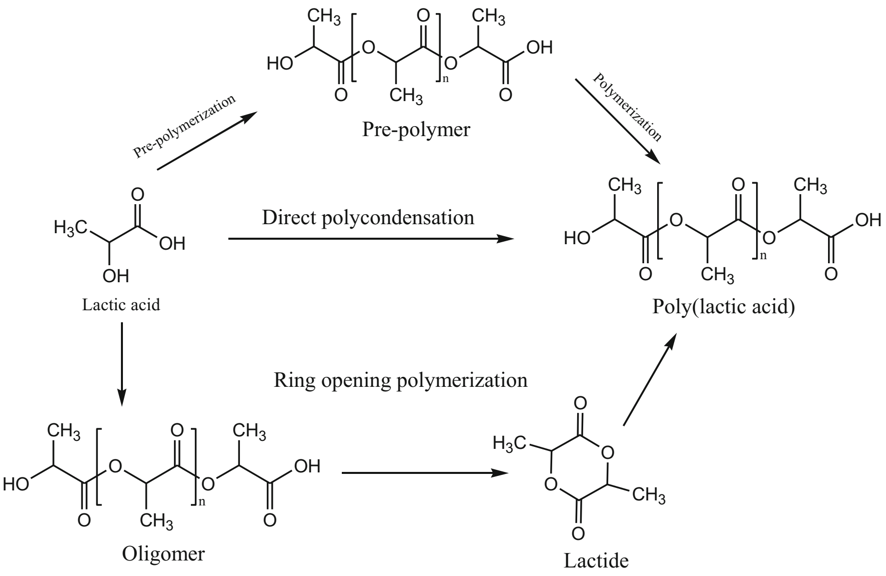

Fig. 17.5 Synthesis of poly (lactic acid) by ring opening polymerization and by direct polymerization

produce lactide through internal transesterification followed by ring opening to give high molecular mass PLA (Lasprilla et al. 2012; Hu et al. 2016).

PLA is a semi-crystalline polymer having a glass transition (T_g) of 55 °C and a melting point (T_m) of 180 °C. It is mechanically stronger than traditional polymers like poly(ethene), poly(styrene), and poly(propylene) (Hamad et al. 2015). PLA is advantageous due to its renewability, processability, biodegradability, biocompatibility, recycling, and compostable nature (Farah et al. 2016). However, it has some disadvantages too like poor toughness (or high brittleness), slow degradation rate (dependent upon the crystallinity), hydrophobic nature (Liang et al. 2017), and chemically inertness due to lack of reactive side chains. Some useful applications of PLA in the biomedical field require its improved mechanical and physicochemical properties including surface roughness, hydrophilicity, and reactive functionalities. Any improvement in hydrophilicity and enhanced chemical functionality would make PLA useful for biomedical applications (Tyler et al. 2016). To improve the biocompatibility of PLA-based implants with innate immune cells, some alterations in its surface properties would be required (Stankevich et al. 2015). For instance, plasma treatment of PLA improves its hydrophilicity by inserting some polar groups. This makes the applications of PLA in the packaging and biomedical field more achievable (Turalija et al. 2016).

Likewise, aminolysis is another approach that creates reactive amine groups on the surface of the PLA matrix. Zhu et al. (2004) used 1,6-hexanediamine to aminolyze the PLA surface which was then immobilized with some biocompatible macromolecules like gelatin, collagen, and chitosan. In another study, photochemical-grafting (using UV light) was employed as a low-cost approach to modify the PLA surface (Ma et al. 2000). Oxygen plasma treatment was also employed to improve the tensile strength of the PLA matrix which was then used to prepare nanofibrous scaffolds (Park et al. 2007). Likewise, air plasma treatment of PLA enhances the surface energy of PLA film, thus improving its adhesion properties (Jordá et al. 2014). The surface roughness and hydrophilicity of PLA films could also be improved via alkaline or acidic hydrolyses (Guo et al. 2015). Table 17.1 summarizes different types of hydrophobic biopolymers, their modification approaches, and applications in various sectors of science and technology.

17.2.2.7 Polyhydroxyalkanoates

PHAs are renewable, biodegradable bioplastics produced by numerous microbes including the bacterial strains of *Azobacter beijerinckii*, *Alcaligenes latus*, *Bacillus megaterium*, *Ralstonia eutropha*, *Pseudomonas oleovorans*, and *Pseudomonas aeruginosa*, some fungi, and the archaea (Volf and Popa 2018). PHAs are stored as intracellular cytoplasmic inclusions, generally, in carbon-rich environments with limiting levels of oxygen, nitrogen, magnesium, and phosphorus in the culture media (Gómez Cardozo et al. 2016; Raza et al. 2018). They occupy the molecular masses ranging from hundreds to thousands. PHAs are structurally diverse, biodegradable, biocompatible, and thermoplastic materials suitable for a wide range of applications in marine, agriculture, and medical fields (Yang et al. 2015).

A PHA molecule may contain 600–35,000 hydroxy fatty acid monomeric units. Each monomer has a side chain (−R group) which may be saturated, unsaturated, branched, or substituted alkyl groups. They are classified based on the number of carbon atoms in the PHA monomer unit, i.e., short-chain length PHA (scl-PHA) consisting of 3–5 carbon atoms, medium-chain length PHA (mcl-PHA) having 6–14 carbon atoms, and long-chain length PHA (lcl-PHA) having more than 15 carbon atoms (Abid et al. 2016; Raza et al. 2018). They have ester bonds to make them hydrophobic (Muhammadi et al. 2015). Therefore, the functionalization of PHAs is required to tune their reactivity via physical and chemical modifications to improve desirable properties for sustainable industrial and medical applications (Raza et al. 2020). PHAs are more advantageous than other bioplastics like PLA, poly(glycolic acid) (PGA), and poly(DL-lactide-co-glycolide) (PLGA), 3-hydroxybutyric acid (3HB), or 4-hydroxybutyric acid (4HB). They have less acidic character than the α-hydroxy acids of PLA, PGA, and PLGA and are finally eliminated from the human body in a short period (Luef et al. 2015; Ali and Jamil 2016).

Cell-matrix adhesion of PHA surfaces could be stimulated through several surface modification approaches like ion implantation, alkaline hydrolysis, lipase treatment, and oxygen, ammonia plasma treatments (Pompe et al. 2007). Surface modification of PHAs via UV irradiation, surface hydrolysis, grafting, or lipase treatment is useful in enhancing their hydrophilicity and biocompatibility (Lu et al. 2013). Poly(hydroxybutyrate) (PHB), the first and most common bio-polyester of the PHAs family, is biosynthesized in numerous bacterial genera like *Staphylococcus*, *Alcaligenes*, *Bacillus*, *Pseudomonas*, *Micrococus*, *and Rhodococcus* under specific fermentation conditions (Ali et al. 2017). PHB consists of

Table 17.1 Hydrophobic biopolymers, their modification, and applications

Polymer	Functional group	Modification technique	Induced functional group	Application after modification	References
Chitin	Acetyl amine ($CH_3CONH–$)	NaOH and amidoxime treatment	Free amine group	Extraction of uranium from seawater	Barber et al. (2014)
Chitosan	Amino group ($–NH_2$)	Nitrogen and oxygen plasma treatment	Oxygen- and nitrogen-containing groups	Enhanced cell attachment and proliferation	Luna et al. (2011)
Chitosan/PEO nanofibers	Amino group	Air dielectric barrier discharge plasma	Increased hydroxyl, –C=O, NH^{3+} polar groups	Improved hydrophilicity for immobilization of acetylcholinesterase	Dorraki et al. (2015)
Silk fibroin nanofibers	Amide	Microwave-induced plasma		Increased hydrophilicity and human articular chondrocytes attachment and proliferation	Baek et al. (2008)
		Oxygen plasma treatment	Hydroxyl, carboxylic groups	Increased hydrophilicity, cell attachment increased, skin regeneration	Jeong et al. (2009)
PCL	Ester	Argon plasma treatment	Carboxylic groups	Increased hydrophilicity, higher cell proliferation	Suntornnond et al. (2016)
		Oxygen plasma treatment	Functional groups containing oxygen	Increasing hydrophilicity, human umbilical endothelial cell adhesion was increased	Recek et al. (2016)
PLA	Ester	UV irradiation	Carbonyl	Surface energy increased as well as hydrophilicity to improve dyeability of PLA films with cationic dyes	Koo and Jang (2008)
PLA foil	Ester	DBD plasma (dry air, helium, argon)	–C–O, –C–N, the carboxylic group increased	Increased hydrophilicity (decrease in contact angle) to improve initial cell adhesion	Jacobs et al. (2013)
		Atmospheric plasma treatment	Insertion of oxygen-based polar groups (–OH, –C–O–O–H, –C–O–C–)	Increased hydrophilicity for improved adhesion	Jacobs et al. (2013)

(continued)

Table 17.1 (continued)

Polymer	Functional group	Modification technique	Induced functional group	Application after modification	References
		Enzyme (Humicola insolens cutinase) (Candida antarctica lipase B) treatment	Increase in number of hydroxyl and carboxylic groups	Increased hydrophilicity for further targeted drug delivery	Pellis et al. (2015)
PHB	Ester	UV irradiation for photografting of PEG	Improved toughness after grafting	Increase in hydrophilicity (decrease in contact angle) to graft PEG on the PHB surface. PHB/PEG composite used for tissue engineering	Cai et al. (2004)
		Alkaline treatment	Increased carboxylic groups	Enhanced proliferation of human osteoblast, inhibit *S. aureus* growth	Karahaliloğlu et al. (2015)
		Lipase treatment	–COOH	The pore size of surface decreased, enhanced proliferation of mouse fibroblast cell line L929	Zhao et al. (2002)
PHBV	Ester	Treatment with Cutinase Tf Cut2 from *Thermobifida fusca* KW3	–COOH	Increased surface hydrophilicity	Berezina et al. (2015)
PHBHHx	Ester	Lipase treatment	–COOH	Increased hydrophilicity and smoothness, enhanced L929 cell growth	Zhao et al. (2002)
		UV irradiation	N.A.	Increased hydrophilicity, brittleness increased, enhanced L929 cell growth	Shangguan et al. (2006)
		Grafted by maleic anhydride	–COOH	Hydrophilicity improved, (enhanced L929 cells and HMECs adhesion and proliferation)	Li et al. (2008)

3-hydroxybutyrate monomers (having 4–5 carbon atoms) and methyl as side groups (Ross et al. 2017). PHB is renewable, biodegradable, hydrophobic, and thermoplastic polyester (Jiang et al. 2015; Raza et al. 2020). Due to its crystalline nature, it is brittle, hydrophobic, and thermally unstable with low melt temperature which limits its uses in biomedical applications (Bhattacharjee et al. 2016). However, the addition of some plasticizers and blending with other degradable polymers can improve its flexibility for multipurpose applications (Jost and Kopitzky 2015).

PHB has been utilized for several biomedical devices like sutures and drug delivery systems. It is highly biocompatible with blood and tissues and is non-toxic as its degradation product (being 3-hydroxybutyric acid) is an innate component of blood (Wani et al. 2016). It has attracted a lot of attention in the biomedical field, but due to its poor hydrophilicity and toughness, surface modification is needed for its full utilization (Cai et al. 2004). It can be made suitable for injectable implant systems, but its surface hydrophobicity can become a hurdle. The water contact angle for such applications should be between 60 and 80°. Different treatments like plasma, alkaline, and hydrolytic enzymes (like lipases) can be applied for improving its hydrophilicity as well as biocompatibility. However, the plasma and alkaline treatments have some limitations; the former results in the aging of polymer (i.e., the hydrophilicity decreases over time), whereas the latter damages the polymer surface (in the form of asperities). Contrary to these limitations, enzymatic treatment is applicable under mild reaction conditions. For instance, a cutinase could be used to surface modify the ester linkage of bio-polyesters via partial hydrolysis (Berezina et al. 2015). The plasma treatment of PHB could also make it hydrophilic by introducing oxygen-containing polar groups onto the polymer surface (Slepička et al. 2015).

17.3 Surface Modification of Biopolymers

Biopolymers find vast applications in the fields of biomedical in the form of adhesives, protective coatings, composites, lubricants, thin-films, and biosensing devices. Thereby, tunning the surface properties of biopolymers based on polarity, chemical composition, crystallinity, roughness, conductivity, and lubricity could be inevitable for their successful applications. Among various biopolymers, the biodegradable aliphatic polyesters had extensively been used in tissue engineering scaffolds due to their good mechanical properties degradation rates and being non-toxic (Shen et al. 2007). Nevertheless, low surface energy and hydrophobicity make these biopolymers weak candidates for cell adhesion and propagation (Morent et al. 2010). The biopolymers' inherent surfaces are mostly not suitable for desirable applications. The purpose of surface treatments has been to improve/impart the functionality of/to the selected biopolymer surface and subsequently provide a reaction site for the approaching molecular species (Yao et al. 2008). The other routes may be tuning the surface energy, chemical affinity, surface cross-linking, and surface morphology to optimize the hydrophobic-hydrophilic balance (Desmet et al. 2009).

Several surface treatments have been established to modify the physical and chemical properties of polymer surfaces without disturbing their bulk properties (Desmet et al. 2009). The biocompatibility of polymer implants can be enhanced by coating their surfaces with proteins or polysaccharides as desirable. For example, heparin, chitosan, or insulin is bound on membranes based on poly(acrylonitrile) to increase their compatibility to blood cells (Wang et al. 2007). The protein attachment improves after making the biopolymer surface hydrophilic (Nath et al. 2004). These surface modification techniques can be divided into three routes including physical, chemical, and biological ones in tailoring the surface properties of biopolymers as needed. The conventional approaches involve the treatment of polymer surfaces with corona, flame, plasma, electron beam, photons, ion beams, γ-rays and X-rays, and so to introduce new functionalities to enhance the surface wettability (Desmet et al. 2009). Various surface modification techniques for PHAs and related biopolymers have been illustrated in the following subsections.

17.3.1 UV Irradiation

The UV irradiation modifies the surface of biopolymers through exciting or oxidation of surface molecules during which chain breakage and cross-linking take place (Kaczmarek et al. 2002). The incident UV photons of energy 3–124 eV and the corresponding wavelengths from 10 to 400 nm can cause chemical and structural changes in the polymers. This results in increasing the amount of oxygen-containing polar groups at the polymer surface, making it more wettable hence promoting free radical grafting of bioactive compounds (Zhang et al. 2008). For instance, the UV irradiation (at 254 nm) of films made of the blends of silk fibroin, collagen, and chitosan resulted in increased hydrophilicity as indicated by contact angle analyses (Sionkowska 2014). Recently, a UV/ozone treatment has been applied on micro-carriers of PCL (of size 150–200μm) to create their oxygen-containing functionalities (like –COOH, –OH) for increased hydrophilicity. The treated PCL micro-carriers immobilized with gelatin exhibited better adhesion and growth of human skin fibroblast cells (HSFC) than with control PCL micro-carriers (Samsudin et al. 2018). The process of UV irradiation on PCL is shown in Fig. 17.6a.

17.3.2 Photo-Oxidation

The oxidation reaction induced due to the presence of light is called photo-oxidation. Sometimes, hydrogen peroxide is also employed in the presence of UV light to boost the surface oxidation/modification of biopolymers (Guan et al. 2000). The UV irradiation may also initiate graft polymerization to extend the surface treatment of biopolymers (Desai and Singh 2004). For instance, PLA is hydrophobic and chemically inactive due to a lack of reactive side chains. So, its surface may be functionalized by introducing some reactive groups like –OH, –COOH, etc. via photo-oxidation (Fig. 17.6b). The surface of PLA could also be modified via

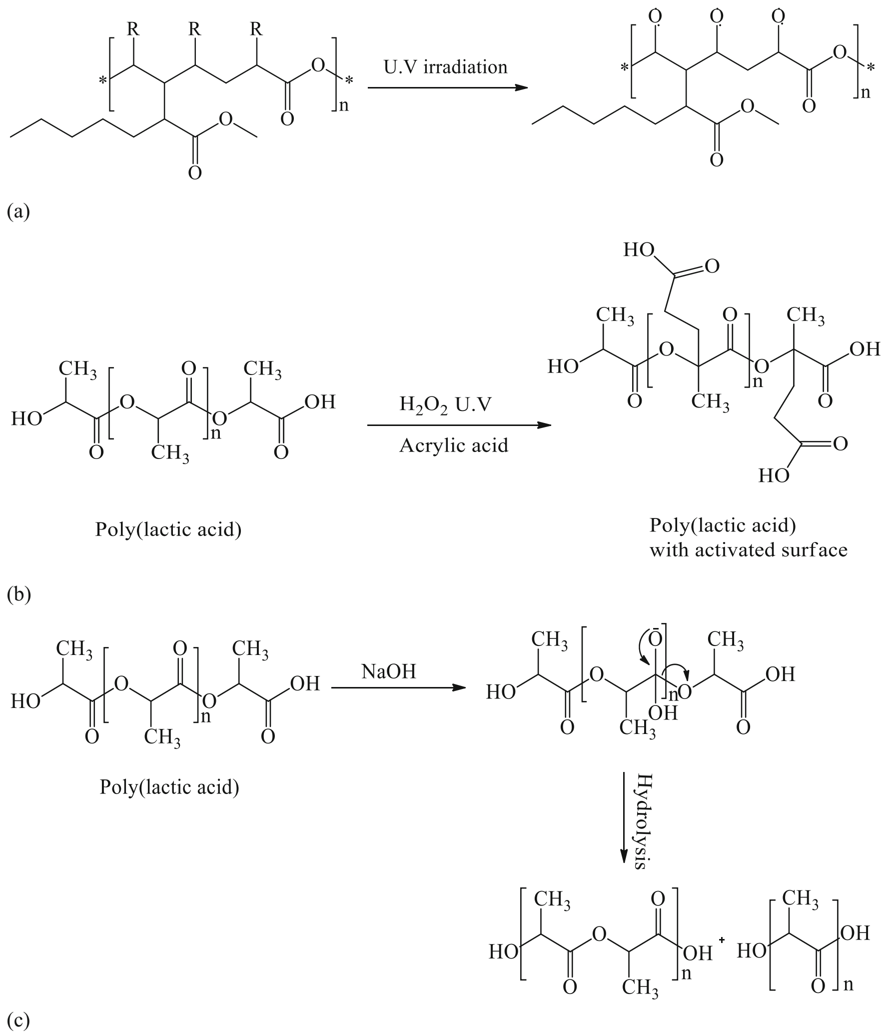

Fig. 17.6 Mechanisms of (**a**) UV irradiation on PCL, (**b**) photoxidation of PLA, and (**c**) alkaline treatment of PLA

photo-oxidation to attach acrylic acid units in the presence of hydrogen peroxide thus using it for cell adhesion and proliferation (Martínez-Carmona et al. 2017).

17.3.3 Corona Treatment

Corona is a glow around a conductor at a high electrical potential. During the corona treatment, a high voltage is applied across the electrode to ionize the air. High energy electrons are bombarded on the polymer surface to break down its surface molecular networks. This results in the production of free radicals which originate cross-linking. They also react with oxygen to introduce oxygen-based functionalities like –OH, –CO, –COOR, –C–O–C, and –COOH on the polymer surface. The polar groups so created during the corona oxidation stay on the polymer surface making it hydrophilic thus enhancing the opportunities for the attachment of polar moieties (Desmet et al. 2009; Drelich et al. 2011; Rocca-Smith et al. 2016). For instance, the corona treatment of miscanthus fiber/PLA composite enhanced its thermal stability (Ragoubi et al. 2012).

17.3.4 Alkaline Treatment

The alkaline treatment at a high temperature can also increase the surface wettability of polymers. Likewise, the hydrophilic functionalities of carboxyl and hydroxyl groups are introduced on the polymer surfaces like poly(olefin) and poly(ethylene terephthalate) during their etching with concentrated bases (Drelich et al. 2011). On surface hydrolysis in alkaline media, hydroxide anions (OH^-) hydrolyze the ester linkages of polyesters to give hydroxyl and carboxylic acid as ending groups (Fig. 17.6c). This phenomenon increases the hydrophilicity of polymer film and a better cell attachment occurs (Ang et al. 2006). Likewise, the alkali treatment of PHBHHX film increases the hydrophilicity of the polymer surface by introducing oxygen-containing functional groups which improve its biocompatibility with blood cells (Shen et al. 2010). In a study, alkaline treatment of PLA had been used to introduce acid groups on its surface and then chitosan grafted onto alkaline-treated PLA surface. This resulted in enhanced rat osteoblast adhesion and propagation properties (Cai et al. 2002). In another study, the PLA surface was hydrolyzed under ethanolic alkaline treatment to improve its hydrophilicity and fibroblast cell attachment (Yang et al. 2003; Hassan et al. 2019).

17.3.5 Aminolysis

Another wet-chemical route to introduce functional groups on the polymer surface is the aminolysis using 1,6-hexanediamine in either aqueous or organic media (Raza et al. 2020). This phenomenon produces free amino groups on the polymer surface which facilitate cells to matrix interactions (Fig. 17.7). However, this treatment not only caused loss of mechanical strength and fast degradation rate but also resulted in the wastage of chemicals and irregular surface outlook (Desai and Singh 2004). The introduction of amino groups on the polymer surface increases its hydrophilicity, thus promoting binding of biomolecules to it or neutralizing any acid molecules produced during the degradation of polymer matrices (Zhu et al. 2002). A hydrophilic polymer surface provides better attachment sites for cells as compared to a hydrophobic surface. Some collagen peptides were incorporated into the hydrophilic surface of P(3HB-*co*-4HB) in an in vitro study, thus promoting wound healing in mouse fibroblast cells (Vigneswari et al. 2016).

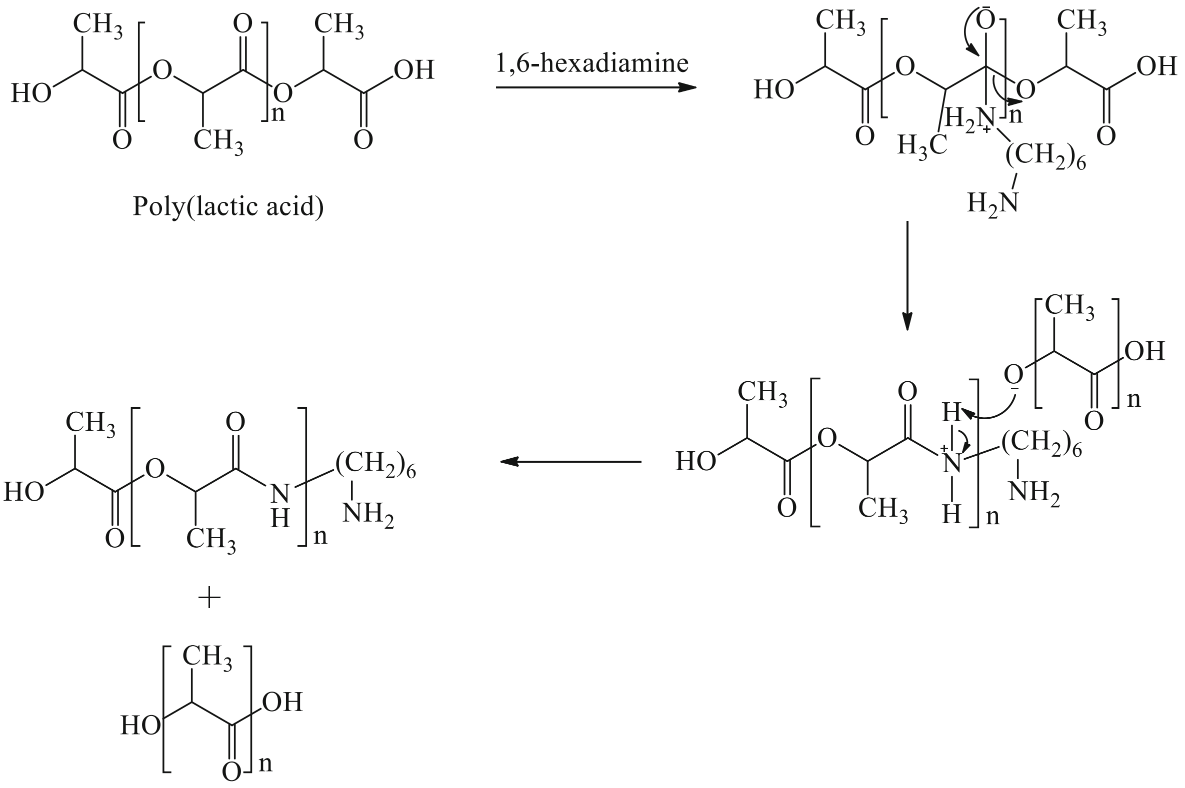

Fig. 17.7 Mechanism of aminolysis of PLA

17.3.6 Graft Polymerization

Graft polymerization is also applicable to modify the polymer surfaces (Fig. 17.8a). The graft polymerization can be accomplished via coordination, ionic, free-radical, or coupling mechanisms. The free-radical mechanism may further be divided into radiation, chemical, and mechanochemical grafting approaches. Different types of

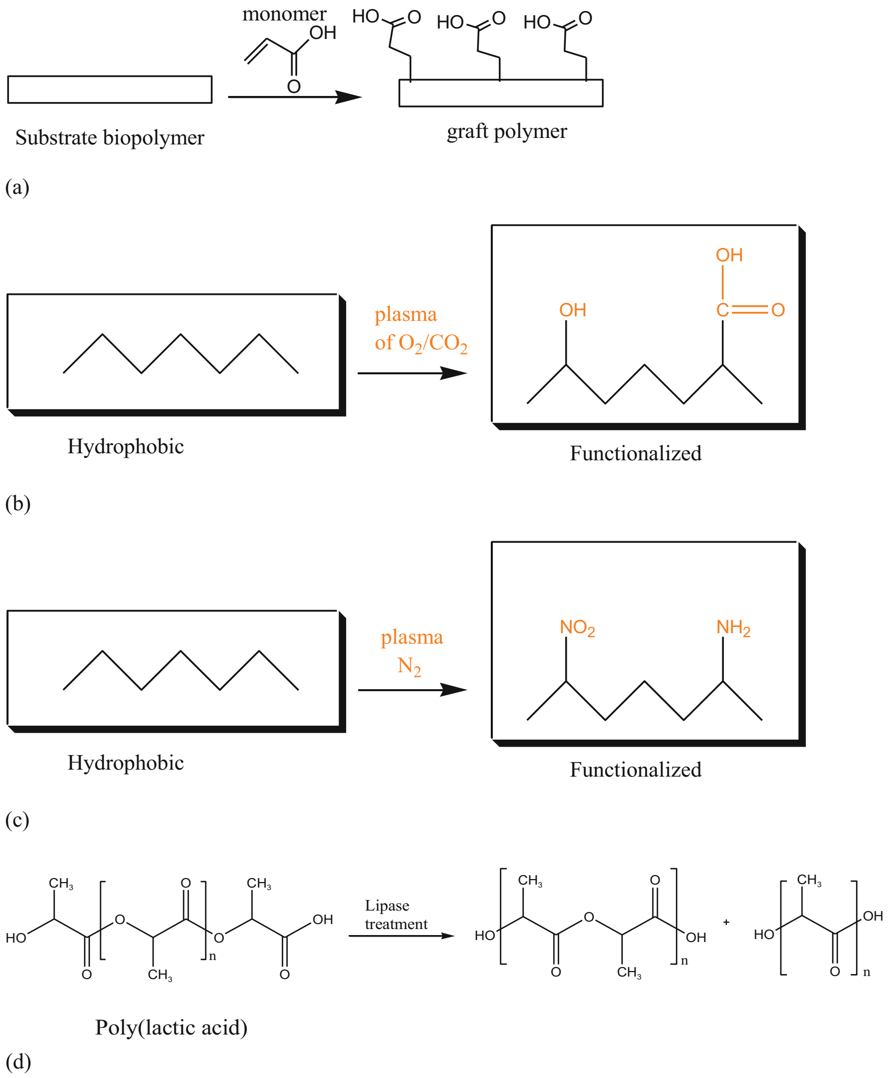

Fig. 17.8 Schematic presentations of (**a**) graft polymerization; plasma treatments for functionalization of (**b**) hydrophilic and (**c**) hydrophobic surfaces, and (**d**) enzymatic treatment of PLA

radiations are used for radiation-induced grafting including X-rays, γ-rays, and electronic rays. Therein high-energy radiations may cause ionization and mid-energy radiations (include laser, plasma, and UV rays), whereas low-energy radiation may bring about required changes in the polymer strength. The process may be controlled using appropriate irradiation energy, dose, and exposure times. For instance, carboxylic acid groups can be grafted onto the PHBV matrix via γ-irradiation. The extent of modification is controlled by the concentration of carboxylic acid (rather than by γ-rays dose) which minimizes γ-ray-induced degradation of PHBV polymer (Grøndahl et al. 2005). Likewise, ozone-treated PHB/PHBV membranes had been grafted with acrylic acid and chitosan (CS)/chitoglucosaccharide (COS). The CS and COS grafted films showed antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), and other *S. aureus* species. The antibacterial activity against *E. coli* was the highest, whereas against MRSA was the lowest against the four test bacteria. Acrylic acid grafting could increase the biodegradability with *Alcaligenes faecalis*, whereas CS and COS grafting reduces the biodegradability. Besides, the CS-grafted PHBV membrane showed higher antibacterial activity and lower biodegradability than the COS-grafted PHBV membrane. Because of the higher surface density of the amino groups, PHBV-CS showed higher antibacterial activity than the PHBV-COS (Hu et al. 2003).

17.3.7 Plasma Treatment

Plasma is so-called the fourth state of matter. The word plasma was introduced by Langmuir in 1929. Plasma is a partially ionized gas containing free ions, electrons, and radicals comprising of neutral atoms or molecules. The surface properties of polymers like roughness, adhesion, wettability, dyeability, and biocompatibility can be modified by inserting polar functionalities on their native inactive surfaces. The effectiveness of modification depends on various factors of plasma treatment like pressure, temperature, gas, intensity, and chemical nature of the test polymer surface (Hoefer 2010). The NH_3 plasma treatment produces amines; O_2 plasma produces COOH and/or OH functionalities; whereas, argon plasma treatment generally introduces free radicals in the plasma chamber. Schematic presentations of plasma treatments of hydrophilic and hydrophobic surfaces are shown in Fig. 17.8b, c, respectively. Herein, an electric discharge ionizes the gas in the plasma chamber at low pressure. The radical gas molecules react with the polymer (like PHAs) and modify its surface characteristics (including mechanical ones) which result in enhanced cell propagation through hydrophilic functionalities (Hoefer 2010). The plasma exposure causes etching of the polymer surface. In low-pressure NH_3 plasma, amine groups were introduced onto PHB thin films without significantly altering the polymer surface morphology (Iu and Eter 2017). Moreover, the cellular attachment had been improved through (oxygen, nitrogen, and microwave) plasma treatment could enhance the surface hydrophilicity and biocompatibility of the PHB and PHBV bio-polyesters (Chang et al. 2018). Further details on plasma treatment can be seen in Table 17.2.

17.3.8 Enzymatic Treatment

The enzymatic modification of plant fibers is becoming important in the textiles and polymers industry. The enzyme-catalyzed reactions are highly specific. Some non-fibrous components from the plant fibers are removed via enzymatic treatments, for instance, removal of lignin from celluloses and gum from the silk. In the case of polymers, enzymes like hydrolases and oxidoreductase had been reported (Lee and Song 2010). Amongst hydrolases: proteases, glycosidases, and lipases, whereas amongst oxidoreductases: laccase, tyrosinase, and peroxidase had been in use for surface modification of textiles and polymers for diverse purposes (Kalia et al. 2013). The schematic diagram for the enzymatic treatment of a bio-polyester is shown in Fig. 17.8d.

For biomedical applications of PHAs, their surfaces have been modified to improve the surface charge, wettability, and hydrophilicity, accordingly. For that purpose, different methods like plasma treatment, alkaline hydrolysis, and lipase treatment had been reported (Guzmán et al. 2011). The enzymatic hydrolysis of PHB changes in its molecular mass, crystal structure, and mechanical strength. The lipase molecules of small size cause biodegradation not only on the surface but also in the interior of the polymer on penetration into it due to surface asperities. The lipase breaks the ester bonds via hydrolysis and produces a lot of hydroxyl groups on the surfaces of both PHB and PLA which increase their hydrophilicity (Artsis et al. 2012). In a study, lipase treatment of PLA fibers could introduce some hydroxyl and carboxyl groups on the PLA surface via hydrolysis of ester linkages (Wang et al. 2009). The PLA membrane surface had been functionalized using cutinase followed by the coupling of human serum albumin (HSA). After surface modification, the PLA exhibited improved antioxidant efficiency and osteoblast cell propagation (Acero et al. 2012; Nyanhongo et al. 2013). Table 17.2 informs the enzymatic treatment of PHB.

17.4 Applications of Biopolymers

17.4.1 Protein Attachment

Proteins are biological macromolecules that are made up of amino acids bonded through peptide linkages. Adsorption of proteins on a surface depends upon its hydrophobicity, surface energy, charge distribution, and texture. The proteins can be manipulated using different methods for their adsorption on the surface of a desirable biopolymer for potential biomedical applications. For instance, bovine serum albumin (BSA) could be adsorbed on the chitosan surface functionalized with different

Table 17.2 Different surface modification techniques applied on hydrophobic biopolymers for potential applications

Surface modification technique	Biopolymer	Functional group	Application after modification	References
Alkaline treatment	PHB	Carboxyl	Improved hydrophilicity, protein adsorption, propagation of human osteoblasts, depressed *S. aureus* growth	Karahaliloǧlu et al. (2015)
	PCL		Early bone formation in rabbit, improved hydrophobicity supported attachment and proliferation of rabbit conjunctival epithelial cells	
Lipase treatment	PHB	N.A.	Increased proliferation of mouse fibroblast cell line L929; reduced surface pore size	Yang et al. (2002)
Air atmospheric plasma	PLA	Carboxyl	Improvement in hydrophilicity	Jordá et al. (2014)
CF_4 microwave plasma	PLLA microfibrous membrane	Oxygen-containing groups increased	Increased hydrophilicity (plasma power100–150 W), have the potential for application w.r.t hydrophilicity	Yue et al. (2015)
Plasma treatment nitrogen, argon, and oxygen plasma (NH_3 and SF_6 plasma)	Chitosan	Oxygen-/nitrogen-containing groups	Increased cell viability, enhance cell spread, and proliferation Increased hydrophilicity with long-term stability	Luna et al. (2011)
	Cellulose			Pransilp et al. (2016)
	PLA/PLLA		Improved hydrophilic property and increase the surface energy of fiber and absorption of ink	Correia et al. (2016)
	Silk fibroin		Improved hydrophilic property and increase the surface energy of fiber Decreased contact angle, increased roughness of film, and	Amornsudthiwat et al. (2013)

(continued)

Table 17.2 (continued)

Surface modification technique	Biopolymer	Functional group	Application after modification	References
			fibroblast cell adhesion	
	PHB		Improvement in L929 mouse fibroblast cell adhesion	Nitschke et al. (2002)
Grafting	Silk grafted with chitosan	Amino	Improved dyeability of chitosan grafted on acylated silk fiber	Davarpanah et al. (2009)
	Silk grafted with L-cysteine	N.A.	The contact angle decreased. Inhibit *S. aureus* growth and control atopic dermatitis	Nogueira et al. (2016)
Photografting of acrylate monomers	PHBHV	–COOH	Antibacterial activity	Manecka et al. (2014)
Aliphatic diamine treatment	Shellac	Amide	Anticorrosive coating for copper protection	Wang et al. (2008)

nanostructured carbons (e.g., GO and CNTs) for enhanced osteoblast attachment (Depan and Misra 2013).

17.4.2 Drug Conjugation

The drug molecules are attached to polymeric molecules and act as drug carriers. Polymers are particularly functionalized with desirable polar groups to enhance the attachment of drug molecules. Thereby, drug-polymer conjugates would be degraded by specific enzymes that appear in an unhealthy tissue thus retarding the risk of damaging the healthy tissues. The enzymatic (like cutinase) treatment of PLA introduces hydroxyl and carboxylic groups on its surface thus increasing hydrophilicity, accordingly. The cutinase-treated PLA surface exhibits more roughness with negative charge thus promoting the loading of positively charged drugs like doxorubicin (Pellis et al. 2017). The enzymatic grafting of carboxylic acids onto the PLA surface was developed using *Candida antarctica* lipase. Moreover, enzymatic hydrolysis of the PLA surface using *Humicola insolens* cutinase increased the hydroxyl and carboxylic functionalities and showed a decrease in contact angle value (Pellis et al. 2015). The alkaline-treated PLA microspheres also behave as hydrophilic for enhanced in vitro cell viability in the rabbit fibroblast cells during targeted drug delivery (Mohd-Sabee et al. 2016).

17.4.3 Cell Adhesion and Proliferation

The aminolysis (using 1,3-ethylenediamine) of PHB creates nitrogen-containing functionalities like –CONH- and/or –$CONH_2$ on its ester groups (Thiré et al. 2007). In a study, poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [P(HB-*co*-HHx)] was functionalized using alkaline and ethylenediamine treatments in aqueous (ED_{aq}) media to improve its porcine urothelial cell adhesion. A minor ED_{aq} treatment significantly improved the hydrophilic character of PHB by introducing amino groups on its surface. Then a YIGSR (Tyr-Ile-Gly-Ser-Arg) peptide was attached to the functionalized substrate for improving the urothelial cell adhesion. The YIGSR peptide tends to inhibit the risk of tumor growth and metastasis (García-García et al. 2013). In a study, the PHBHV surface was modified using oxygen, argon, and nitrogen plasma treatments. It resulted in increased hydroxyl functionality with decreased contact angle and improved cell adhesion (Garrido et al. 2011). In a study, atmospheric plasma treatment and collagen coating of poly(L-lactide-co-caprolactone) (PLCL) film was carried out to make it multiporous for improved proliferation, maturation, and adhesion of human embryonic stem cells derived from retinal pigment epithelial cells (Sorkio et al. 2015).

In another study, HE800, a glycosaminoglycan-like marine exopolysaccharide (EPS), was grafted on the hydrophobic PHA scaffolds for improved adhesion and growth of human mesenchymal stem cells (Lemechko et al. 2013). Likewise, a Gly-Arg-Gly-Asp (GAGD), an adhesive peptide, was photochemically grafted on the chitosan surface to promote the adhesion and proliferation of human endothelial cells (Chung et al. 2002). Photoactivation of electrospun nanofibrous 3-PHA scaffolds promoted attachment of gelatin on it by introducing epoxy functionality, i.e., the hydrophilicity of the PHA scaffold increased. The adhesion of mesenchymal stromal cells, cell proliferation, and osteogenic differentiation were also improved (Grande et al. 2017). The biocompatibility of PHA was improved via lipase treatment to increase the growth of L929 cells on its films, and this treatment was more effective than alkaline treatment (Zhao et al. 2002). The crystalline nature of PHB restricts the adhesion and growth of mammalian cells. The lipase treatment of PHB films increased cell growth 40 times on treated films as compared to untreated films (Kai et al. 2003).

17.4.4 Tissue Engineering

Tissue engineering is the repair or replacement of biological tissues (muscles, skin, blood vessels, bone, etc.) with tissue scaffolds. In the medical field, tissue scaffolds are used for novel viable tissue formation. Often, tissues used for tissue engineering require specific structural and mechanical characteristics for appropriate functions. Tissue engineering also involves the usage of cells in regenerated biobased organs. In a study, braided ropes of chitosan-PLA were used as artificial tendons. The alkaline treatment of PLA fiber partially hydrolyzed the ester linkage on the PLA surface. Then it was treated with chitosan so that the ionic bond was formed between the amino functionality of chitosan and carboxylic functionality of the PLA. Some braided ropes were prepared and tightened by increasing core ropes; chitosan- and alkaline-treated PLA-braided ropes had more hydrophilicity and could be used as artificial tendons (Furuike et al. 2015).

The surface of the electrospun nanofibers of lactic acid was functionalized by introducing an amine group through aminolysis using poly(allylamine). Then epidermal growth (EFG) factor was grafted onto functionalized lactic acid. These grafted scaffolds were able to support neural stem-like cell (NSLC) culture (Haddad et al. 2016). In a study, coaxial electrospinning was used for the fabrication of the PLA/SF/nerve growth factor (PS/N) scaffold in which nerve growth factor (NGF) and SF were encapsulated. Then air plasma treatment was employed to enhance the hydrophilicity of the PS/N scaffold without damaging the nanofibers. Therein, PC12 cells were cultured on both plasma-treated PS/N and untreated scaffolds. The plasma-treated scaffolds supported the adhesion and differentiation of PC12 cells and also exhibited its role as a substrate for nerve tissue engineering (Tian et al. 2015). Likewise, chitosan-grafted SF yarn was subjected to low-temperature oxygen plasma treatment. The modified yarn exhibited improved anti-thrombogenicity and antimicrobial activity against *Bacillus subtilis* and *Escherichia coli* bacterial species. The researchers then impregnated the Penicillin G sodium salt as an antibiotic drug on the modified yarn to improve its surgical applications (Choudhury et al. 2016). In a study, argon and nitrogen plasma treatment of chitosan membranes made the surfaces more roughened with higher surface energy. Therein, nitrogen- and oxygen-containing groups were introduced on the polymer surfaces. The plasma-treated membranes exhibited improved cell viability hence could be used to improve the fibroblast adhesion in tissue engineering applications (Silva et al. 2008).

17.4.5 Miscellaneous Applications

The surface of cellulosic fabric could be modified using oxygen, nitrogen, and sulfur hexafluoride plasma treatments to tune the wettability of the fabric as per requirement for improved dyeability. The oxygen and nitrogen plasma treatments improved the hydrophilicity and surface energy by introducing oxygen and nitrogen-containing functional groups, respectively, whereas, the surface of SF6 plasma-treated cellulosic fabric became hydrophobic causing less ink absorption during inkject printing (Pransilp et al. 2016). The surface of shellac was modified through cross-linking with 1,3-propanediamine. The modified shellac was coated on a copper electrode and dipped in 0.5 M NaCl solution which inhibited the water penetration up to 24 h. After 72 h of immersion, the modified shellac started losing its water penetration capacity due to the introduction of hydrophobic aliphatic groups on the shellac surface (Wang et al. 2008). Likewise, the surface hydrophilicity of starch was enhanced through plasma treatment. This approach has widened the applications of starch in the fields of paper, food, packaging, and pharmaceutical industries (Wiacek 2015). Chitosan is used as a bio-sorbent in wastewater treatment systems due to its amino groups with providing sites from the complex formation with metal ions. So being polycationic, chitosan interacts with anionic species like dyes. In the protonated form, amino groups can interact with the anionic species via electrostatic interactions or the ion exchange. In a non-ionized state (having lone pair of electrons on nitrogen atom), chitosan can interact with electron-deficient species like cations. This phenomenon allows the interaction between chitosan with the hydrophilic species (either cationic or anionic) (Desbrières and Guibal 2018).

17.5 Conclusions and Future Perspectives

Biopolymers with tuneable features may become strong candidates for biomedical applications. To improve their mechanical and surface chemical properties, a lot of strategies have been offered. The surface-modified biopolymers could play better for the said purpose. Besides environmental concerns, both native and modified biopolymers, might be sustainable alternatives to the synthetic polymers. However, the native hydrophobicity of the biopolymer surfaces had been limiting their usage in various biomedical applications. Hereabove, different effective approaches have been presented that could modify the surface of biopolymers for improved hydrophilicity which plays a vital role in biomedical applications. The surface chemical modification methods, however, have some limitations such as disposal of hazardous chemicals into the environment and severe treatments which may lead to unwanted surface alterations. Plasma treatment may be suitable but still is expensive and not feasible on a mass scale. The enzymatic surface modification, however, is an ecofriendly and substrate-specific approach, but it still needs efforts to make it economical. In the future, it is expected that increasing attention would be directed toward the improvement of these modification methods along with the search and development for new sustainable approaches.

It is worth mentioning that many surface-modified biopolymers are still not available at the mass level because of their high production cost, time, and labor requirements. Advanced techniques and scientific skills are further needed to enhance the productivity and usage of hydrophilic biopolymers in various fields. In the future, surface-modified biopolymers would be offered as substitutes for medical implants, controlled structures, and release of bioactive and/or chemical active species for agriculture, textiles, and advance aerospace applications. The specific features of surface-modified biopolymers are to establish hydrophilic-hydrophobic switching as per requirement, for instance, to attach cells, drugs, or proteins when required and to release them on tuning their surface chemistry.

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Multicomponent Polymer Systems Based on Agro-Industrial Waste

18

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This work is dedicated to the memory of Prof. Cristofor I. Simionescu (1920–2007) on the occasion of the commemoration of the centenary of his birth.

Abstract

Nowadays concerns over global warming favored research to focus on the environmental pollution and waste accumulation and on the possibility to recycle and up-cycle the waste. A particular class of such waste consists of natural remains and by-products from agriculture and industries, such as forestry, pulp and paper, furniture, and food industry. There are various methods to upcycle and capitalize the agro-industrial waste: (1) anaerobic digestion for energy production; (2) thermal conversion for the resulting liquids and char that may be further used in obtaining other materials, bioplastics included; (3) industrial processing to obtain natural fibers, long or short, able to be employed as reinforcing materials in biocomposites (multicomponent systems where the matrix consists of polymers or polymer blends, either synthetic or natural; they may also contain emulsifiers, compatibilization agents, antioxidants, as well as inorganic, organic, metal, or metal oxides micro- and/or nanoparticles); and (4) composting. Composites based on agro-industrial waste displayed a level of performance high enough to grant their use in a wide variety of applications:

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furniture and civil constructions; interior design, fencing, and decking; automotive and aerospace industry; food and general packaging. To obtain such materials, it is necessary to consider specific issues, the most important being the compatibility between matrices and reinforcing material. Characteristics of the final material (mechanical, thermal, water sorption, weather resistance) strongly depend on a good compatibility. A critical assessment of the selected methodology is necessary as it is reflected in the final cost of production, which must be limited in relation to the envisaged application.

Keywords

Multicomponent systems · Agricultural waste · Industrial waste · Applications

18.1 Introduction

Modern days concerns over the global warming favored research to focus on the environmental pollution and waste accumulation on one hand and, on the other hand, on the possibility to recycle and up-cycle the waste, in order to response to the societal needs and to replace, at least partially, the classic materials based on fossil resources. One very particular class of re- and up-cyclable waste consists of natural remains and by-products (wood chips and sawdust, straw, husk, stalk and leaves, pulp, etc.) from agriculture and various industries, such as forestry, pulp and paper, furniture, food industry, etc. The chemical composition of such waste varies in correlation with their nature (animal origin—proteins: silk fibroin, wool fibers, egg shells; plant origin—carbohydrates: cellulose, lignin, hemicellulose; polyphenols; etc.) and location (marine organisms—carbohydrates: chitin, chitosan, etc.; proteins—seashells).

There are various methods to up cycle and capitalize the agro-industrial waste: (1) the anaerobic digestion—used mainly for energy production (gas); (2) thermal conversion—the resulting liquids and char may be further used in obtaining other materials, bioplastics included; (3) industrial processing to obtain natural fibers, long or short, able to be employed as reinforcing materials in biocomposites (multicomponent systems where the matrix consists of polymers or polymer blends, either synthetic or natural; they may also contain emulsifiers, compatibilization agents, antioxidants, as well as inorganic, organic, metal or metal oxides micro- and/or nanoparticles); and (4) composting. The wide variety of added-value products (Fig. 18.1) strongly depends on the type of approach, degree of transformation, technology used for processing, range of applications, possibility to include these processes into circular economic loops (recovery and reuse) in order to limit and/or eliminate waste disposal, etc.

In this regard, a more responsible selection and disposal of agro-industrial waste, the employment of knowledge-based, and sometimes state-of-the-art, technologies (single or integrated in synergic combinations), and committed local policies inspired from the regional strategies for the environment protection and preservation

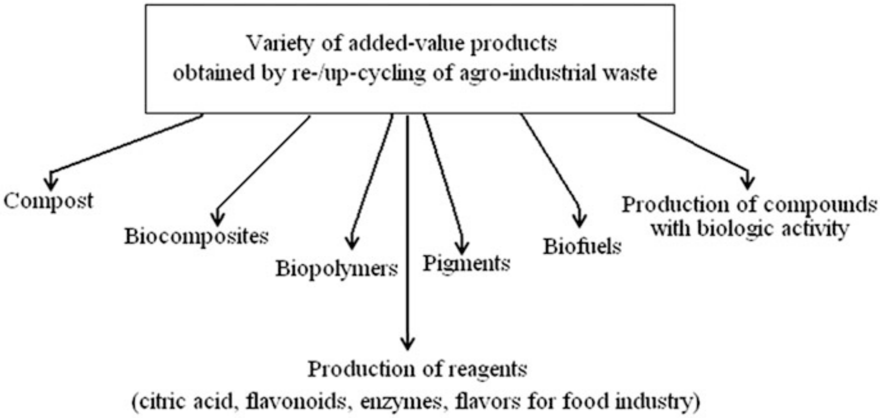

Fig. 18.1 Methods to upcycle and capitalize the agro-industrial waste

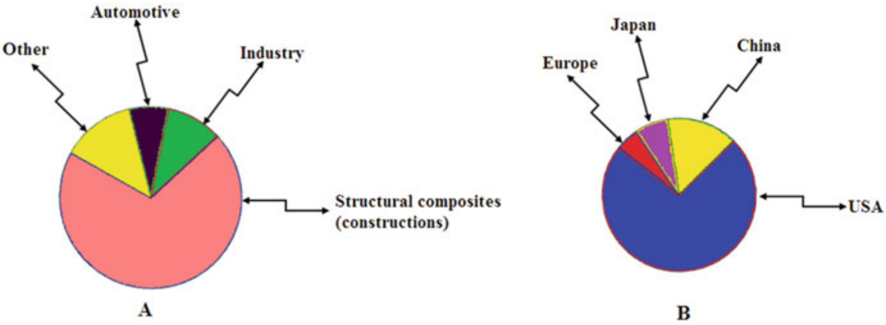

Fig. 18.2 Thermoplastic biocomposites market shares: (**a**) in applications; (**b**) in different countries. (Adapted from Zaaba and Ismail 2019)

are key factors. The market indicators confirmed that structural composites (building panels, railing, fencing, decking, roofing, siding, interior compartmenting) recorded the largest share, based on high volume sales, while industry (automotive industry included) comes the second, despite the knowledge-based development of new products with high added value (Fig. 18.2a). These conclusions are in good concordance with the data for the market shares of different countries (Fig. 18.2b) (Zaaba and Ismail 2019).

Another problem related to this subject is the land management, and it originated in the increasing demand for food and natural fibers. Maintaining a wise balance between production and demand, between land depletion and sustainable agriculture, and all the time preserving the whole ecosystem, may offer tools to provide for both social and economic needs, without sacrificing this natural resource—agricultural land—for the future generations.

This chapter offers an overview of some of the most recent literature data concerning the composites based on polymer matrices and agro-industrial waste as reinforcement, using the type of polymer matrix as classification criterion. Specific problems related to the manufacture of polymer composites based on agro-industrial waste will be presented, as well as some advantages and disadvantages of these composites.

18.2 Manufacture of the Polymer Composites Based on Agro-Industrial Waste

Polymer composites based on agro-industrial waste displayed a level of performance high enough to grant their use in a wide variety of applications: furniture and civil constructions; interior design, fencing, and decking; automotive; sportswear; food and packaging; and adsorbents and filtration media (Berthet et al. 2016; Leao et al. 2010; Pandey et al. 2010; Thakur et al. 2014; Sanyang et al. 2016; Tanasă et al. 2020a). To this purpose, it is necessary to take under consideration some specific issues. The compatibility between matrix and reinforcing material (whether it is fibers, particles or powder) is of utmost importance as it determines the dimensions of the interphase and type and strength of interactions at the interface between components, hence the compatibility and adhesion of components. The characteristics of the final material (mechanical strength, thermal properties, water sorption, UV and biologic resistance) strongly depend on these parameters. Therefore, all aspects of manufacturing these materials must be controlled so as to enable all factors to converge to the desired end products.

Thus, uniformity, consistency, and predictability of composite properties may be reached by using particles/fibers of a specific particle size and dimensional dispersity, but for each type of composite particle size must be wisely considered as some applications require a narrow particle size distribution, while others benefit from the mixture of particles/fibers of different size and sometimes different shapes (Kumar et al. 2017, 2020). Associated to this issue, harvesting, transport, storage, and preliminary preparation steps (e.g., dusting, drying etc.) must also be taken into consideration because these operations will be reflected in the final cost of production.

Another problem that must be addressed is the nature of the polymer matrix employed in these composites manufacture. Literature of the last decades abounds in reports on various composite formulations specifically designed for agro-industrial waste reinforcement. Be they thermoplastics (polyethylene PE, polypropylene PP, polyamides PA, etc.) or thermosets (urea-formaldehyde UF, phenyl-formaldehyde PF, methylene diphenyl diisocyanate MDI, etc.), synthetic or natural or naturally derived (starch, lignin, poly(lactic acid) PLA etc.), and organic or inorganic (gypsum, Portland cement, etc.), the main issue is to choose the most suitable matrix for the selected reinforcement and the envisaged application. Subsequently, the technology must be adapted to the specifications for each type of polymer and reinforcement, whether the end products are particleboards or fiberboards (Abba et al. 2013).

In close relation with these technological aspects are, at the same time, the compatibilization strategies that have to be adopted in a discriminative manner.

Typically, the compatibilization methods may be applied to (1) the polymer matrix (Zănoagă and Tanasă 2011; Zanoaga et al. 2016), (2) reinforcement (Li et al. 2012; Singha and Rana 2012), or (3) both matrix and reinforcement (Pracella et al. 2010; Teaca et al. 2018; Zaaba and Ismail 2019). The surface functionalization of agro-industrial waste intended for polymer composites can be achieved by different means and methods: chemical, physical, mechanical, radiative, etc. (Thakur et al. 2015; Collins et al. 2019; Ferreira et al. 2019; Tanasă et al. 2020b), each of them with advantages and drawbacks, but all aiming at reducing the specific hydrophilicity of natural fibers versus the hydrophobic character of polymer matrices and, by consequence, increasing the dimensions of the interphase and improving the interfacial interactions. Hence, a better compatibility of components and a higher level of performance of composites can be achieved.

Nevertheless, properties other than mechanical ones can be also improved by using different additives in composite formulation. Thus, resistance toward water and humidity can be achieved by particle/fiber functionalization, as well as by adding natural (rosin, waxes) or synthetic resins, which may act at the surface of the composite or in-depth (Zach et al. 2014; Brzyski et al. 2017; Cichosz and Masek 2019). Hydrophobization has proven to be important not only for composites used in outdoors applications, but indoors too, as it grants composites dimensional stability due to a limited adsorption of water. Thus, crack formation, and subsequently mechanical failure, is significantly delayed. Even more, composites with increased resistance toward water and humidity have, at the same time, a good resistance to weathering and biologic attack when used in outdoor applications. Flame retardants may also be included in these composites formulation in order to render them improved thermal stability and flame resistance. Some of these additives are inorganic compounds (Sain et al. 2004); others are organic products or polymers (Subasinghe and Bhattacharyya 2014; Toldy et al. 2017; Elsabbagh et al. 2018). Depending on the chemical structure of these additives and their heteroatom, they can act in different stages of the burning process and in different phases as well (gas, liquid, or solid). Halogen-containing compounds act as gas with flame inhibiting effect, while phosphorus-based agents reduce the formation of flammable vapors by increasing the char formation upon pyrolysis (Sain et al. 2004).

Weathering, photochemical degradation, and biologic attack must be also considered when it comes to manufacturing composites made of polymers and agro-industrial waste. Their behavior upon UV radiation and under complex weathering conditions (variation of temperature, seasonal or freeze–thaw cycles, humidity, pH, pollutants, ozone, microorganisms, wind, solid particles in air) is of high importance in terms of functional reliability and service life (Zanoaga and Tanasa 2016; Teaca et al. 2019). After addressing these problems, multicomponent polymer systems with complex composition result at the end of the technological process. The wise selection of components and processing approach enables them to act in synergy as to respond to requirements of the envisaged applications.

18.3 Synthetic Polymer Matrices

Synthetic polymers reinforced with agro-industrial waste still retain the interest of scientists in both fundamental research and industrial applications because these materials offer certain possibilities for the waste management and allow energy and material recovery. The most commonly used synthetic polymers for the manufacture of such composites are thermoplastics (polyethylene PE, polypropylene PP, polyamides PA, polystyrene PS) and thermosets (urea-formaldehyde UF, phenyl-formaldehyde PF, methylene diphenyl diisocyanate MDI, epoxydic resins, unsaturated esters). Some illustrative data regarding the main characteristics of thermoplastics as compared to thermosetting polymers are summarized in Table 18.1.

18.3.1 Thermoplastic Matrices for Composites with Agro-Industrial Waste Reinforcement

18.3.1.1 General Considerations

Thermoplastic polymers are highly appreciated as matrices for a wide variety of composites and biocomposites, but thermoplastics with low melting temperatures are particularly preferred for composites with natural fillers or reinforcements, such as wood chips, shives of flax flakes, cereal straws, corn and cotton stalks, olive wet/dry husk and pits, tomato peels, grape stalks and pomace, textile fiber waste, etc. (Kozlowski and Wladyka-Przybylak 2004; Thakur et al. 2014; Navas et al. 2015; Zaaba and Ismail 2019). Typically, polyolefins (PP, PE) and polyamides (PA,

Table 18.1 Main properties of thermoplastics versus thermosets[a]

Polymer	Density (g/cm^3)	Melt temperature (°C)	Tensile strength (MPa)	Elastic modulus (GPa)	Elongation at break (%)
Thermoplastic polymers					
PE	0.91–0.96	105–140	15–78	0.055–1.5	2–800
PP	0.90–0.92	160–176	26–41	1.0–1.8	15–700
PS	1.04–1.06	110–135	25–69	4.0–5.0	1–3
PVC	1.40	100–260	41–52	2.8	40–80
Copolyamide[b]	1.11	125–135	20	1.1	300
Thermosetting polymers					
Polyester	1.0–1.5	90–250	40–90	2.0–4.5	< 2.6
Vinylester	1.2–1.4	86	69–86	3.1–3.8	4–7
Epoxy	1.1–1.6	100–200	28–100	3.0–6.0	1–6
Phenolic	1.29	210–360	35–62	2.8–4.8	1.5–2.0
Polyurethane	1.04–1.46	–	41–90	2.1–4.4	1–3

[a] Adapted from Ku et al. (2011), Beyler and Hirschler (2001), Holbery and Houston (2006), Bogoeva-Gaceva et al. (2007)

[b] Zanoaga and Tanasa (2002)

mainly aliphatic or mixed aliphatic-aromatic PA, with low melting point, and having properties as hot-melt adhesive, if possible (Zanoaga and Tanasa 2002; Zanoaga et al. 2016)), polyvinyl chloride (PVC) (Wang et al. 2008; Zainudin et al. 2009), polystyrene (PS), or even polymer blends (Clemons 2008) are widely considered. In this regard, the most sensitive issue is the processing temperature which usually has to be maintained under 200 °C to prevent the thermal degradation of the natural fillers (Väisänen et al. 2016). Compared to thermosets, thermoplastics offer some benefits, such as the possibility to recover and re-use them (up to a certain extent and with minimum property decay) (Bourmaud and Baley 2007). Even more, in certain cases the matrix is made of polymer waste, if appropriate, from the very beginning (Zănoagă and Tanasă 2011). In terms of mechanical properties, thermoplastic matrices afford tensile strength values comparable with the thermosets, but the elasticity of these composites is definitely superior. Given the hydrophilic nature of agro-industrial waste and their ability to absorb water even when included in plastic composite formulations, the poor compatibility with the matrices is obvious. Compatibilization strategies were designed and successfully applied, as follows:

- Functionalization—(1) surface functionalization of fillers and (2) polymer functionalization—refers to various methods to chemically and/or physically alter one or both components of the composite (Dányádi et al. 2010; Moghadamzadeh et al. 2011; Tanasă et al. 2020b);
- Use of coupling agents which are low molecular/oligomeric reagents used to promote chemical bonds formation between matrix and filler, as well as hydrogen bonding, and polymer entanglement; in the case of polyolefins, the most commonly used are maleated (maleic anhydride MA) oligomers (PP-*g*-MA, PE-*g*-MA); other coupling agents are monofunctional isocyanates, *m*-phenylene bismaleimide, etc. (Mohanty et al. 2006; Väisänen et al. 2016; Zanoaga et al. 2016; Tanasa and Zanoaga 2018).

  From technological point of view, most industrial equipment and technologies employed for processing thermoplastics can be used to manufacture thermoplastic composites with natural fillers. Among other parameters, the mixing regime has to be carefully considered as it can negatively influence the properties of the final product, as follows:
- The use of one screw mixer/blender might be ineffective in terms of distribution of filler inside the matrix, even at high speeds, because it may cause secondary agglomeration of the filler and cannot mobilize the material stuck in the blind spots of the machine.
- If a twin-screw (co-rotating or counter-rotating) extruder is employed, the speed must be preset so that no mechanical degradation of polymers will occur due to the high values of the shear force created between screws (especially for the counter-rotating screws).
- Although the fact that co-rotating extruders provide better mixing than the counter-rotating extruders is still under debate, it was experimentally proved the counter-rotating twin-screw extruders generate a higher-pressure build-up,

so they may be preferred over co-rotating twin-screw extruders for profile extrusion (Shah and Gupta 2004).

As a direct result of the combined effects of all the above-mentioned factors, the main properties of the final composites get improved.

Mechanical properties: Typically, natural particles and fibers have a reinforcing effect so that an increase in tensile strength and elastic modulus is evident (El-Saied et al. 2012). Tensile strength is more sensitive to the polymer matrix, while the elastic modulus depends mainly on the reinforcement. Therefore, strong interfacial interactions (adhesion and cohesion forces) are the condition for the composite to reach an increased tensile strength, along with orientation of fibers (when required) and a low concentration of stress centers. On the other hand, higher values of tensile modulus are achieved at high amounts of filler and when the polymer matrix is wetting very well the filler particles, and a large interphase results. The aspect ratio of particles/fibers strongly influences the fracture properties of the composite: the higher the ratio, the better the fracture behavior (Nourbakhsh and Ashori 2010; Viksne et al. 2010).

In principle, there is a minimum critical length of the particles/fibers for the composite to reach high performance mechanical properties. If the length of the filler is less than that, the interphase is not strong enough as the particles cannot create a co-continuous phase within the matrix, and debonding occurs regardless the nature of the thermoplastic. When the length exceeds the critical length, the composite may become brittle (especially at high amounts of reinforcement) because the matrix cannot wet satisfactorily the particles/fibers and the system becomes rigid (Tanasă et al. 2014). Secondary alteration of fibers (curling, bending) may also occur (Väisänen et al. 2016). At this point, the impact strength of the composite is compromised.

Water sorption: The amount of water absorbed by a composite reinforced with agro-industrial waste strongly depends on the type and nature of the reinforcement. Thus, eggshells and seashells (Owuamanam and Cree 2020) will cause a much lower water sorption than feathers (Bullions et al. 2006) and wood fibers or particles, rice husk, or kenaf fibers (Tajvidi et al. 2006). When referring to natural fiber-reinforced thermoplastics, an almost linear correlation was found between the amount of filler and the water sorption of the composite (George et al. 1998), but the fiber size is important as well, as it contributes to transfer water molecules from the surface to interior and inside the composite by diffusion from fiber to fiber (Migneault et al. 2008). This is, in fact, the dominant mechanism of water sorption in composites with high load of reinforcement, when large bundles of filler stick together. When the filler content is low, then the mechanism is percolation (Wang et al. 2006).

Thermal characteristics: The thermal degradation of polymers reinforced with natural fillers evolves in stages. It starts with the loss of water linked physically and chemically onto the filler surface, continues with the decomposition of the natural filler, and the final stage being the thermal degradation of the polymer matrix (Monteiro et al. 2012; Stokke et al. 2014). When thermal degradation of filler is initiated during the service time, the decay of other properties takes place in avalanche: mechanical properties weakened, the exterior aspect is no longer esthetic, bad odors develop under biologic attack, etc. Moreover, the thermal stability and flame resistance of these composites must be considered from different standpoints, such as: (a) type and nature of filler—for example, flax fibers have long ignition time and reach the flashover point very slowly (Manfredi et al. 2006); (b) addition of flame retardants (Chapple and Anandjiwala 2010).

Some of the most recent reports on thermoplastic composites with agro-industrial reinforcements will be reviewed in the following section, using as criterion the nature of the polymer matrix.

18.3.1.2 Thermoplastic Matrices for Composites with Agro-Industrial Waste

Polyolefins: PP and PE are the most commonly used thermoplastics in composite formulations. In any form, either, freshly prepared polymers or recovered waste, or high density or low density polymers, with or without a coupling agent, or employed in either structural or non-structural, indoors or outdoors applications, PP and PE showed a versatility that makes them suitable candidate for agro-industrial waste for reinforcement. Some illustrative examples are summarized in Table 18.2.

Polystyrene (PS): PS is a synthetic, inexpensive, relatively low melting point aromatic polymer, with poor barrier properties toward oxygen and water vapor; it is non-biodegradable, but easy to process by extrusion (Styrofoam), molding, and vacuum forming. Composites based on PS and agro-industrial waste showed good mechanical and thermal properties, as presented in Table 18.3.

Polyvinyl chloride (PVC): PVC is the world's third most widely produced synthetic plastic polymer (after PE and PP); it comes in two basic forms: rigid (RPVC—used for pipes and in profile applications, such as doors and windows) and flexible (by the addition of plasticizers). In composites with cotton or linen, it is used for the manufacture of canvas. Composites with agro-industrial waste as reinforcement were prepared using various additives, such as compatibilizers, impact modifiers, and lubricants, as summarized in Table 18.4.

Polyamides (PA) and co-polyamides: Polyamides and co-polyamides are a viable alternative to polyolefins as matrices in composites reinforced with agro-industrial waste (Taj et al. 2007). Their lower hydrophobic character as compared with polyolefins favors the matrix-filler interfacial bonding, even without adding compatibilizers. Still, there are other processing difficulties due to their higher melting temperatures, so the composite manufacturing must be adapted as to avoid the thermal degradation of natural reinforcement. Some examples are presented in Table 18.5.

Thermoplastic polymer blends: Composites with agro-industrial waste were produced using thermoplastic polymer blends as matrix as well. Various combinations were considered, sometimes even incompatible polymers, and applied with satisfactory results, as summarized in Table 18.6.

Most noticeable improvements were recorded for the mechanical properties of composites, but thermal stability and flame retardancy and resistance to water increased as well upon the addition of coupling agents and/or other selected

Table 18.2 Thermoplastic composites with polyolefins as matrix

Polymer matrix	Agro-industrial waste/additive	Manufacturing method	Results/ observation	References
Polyethylene (PE)				
Recycled PE	• 20–39% wood sawdust • 3 wt% Exxelor VA 1803 as compatibilizer.	Extrusion and compression method	The wood sawdust in the PE matrix decreased the thermal stability of the blends	Constantinescu and Cazacu (2007)
HDPE	• 20, 40, and 60 wt% wood flour • 20, 30, 40, and 60 wt% paper sludge • 2 and 3 wt% coupling agent (PE-g-MA)	Co-rotating twin-screw extruder, granulated and injection-molded samples	Tensile modulus and flexural properties of the composites increased by the addition of the sludge and MAPE, while water absorption and thickness swelling decreased	Hamzeh et al. (2011)
HDPE	• 30 wt.% wood flour • 0, 2, and 4 wt.% modified montmorillonite •3 wt.% PE-g-MA as coupling agent	Injection molding	The addition of nanoclay provided indirect protection against weathering, little decrease in modulus of elasticity due to low water absorption, and reduced oxidative action	Eshraghi et al. (2016)
RecycledPE	•30–40% waste: cacao husks, beet pulp, husks of buckwheat, rice, millet and sunflowers, and potato and maize pulp (maximum size of the filler particles was ≤150μm) • 2% bentonite as dispersing agent • 2% activated carbon as sorbent	Extrusion and compression molding	Composites with cacao husks, beet pulp, and rice husks showed good mechanical properties	Kirsh and Chutkina (2011)
LDPE	• 4 wt% rice husk • 6 wt% MAPE • 0–6 wt% MMT	Extrusion blown films	Good mechanical properties for a	Majeed et al. (2017)

(continued)

Table 18.2 (continued)

Polymer matrix	Agro-industrial waste/additive	Manufacturing method	Results/ observation	References
			variety of food packaging	
LDPE	• 2, 5, and 7 wt.% rice husk • 2 wt% MMT	Extrusion blown films	– Increased tensile modulus – Decreasing tensile and tear strength	Majeed et al. (2017)
LDPE	• Rice husk • MAPE • 0–6 wt% MMT	Extrusion blown films	Improved tensile strength and tear strength, O_2 barrier properties, and thermal stability	Majeed et al. (2014a)
LDPE	• 5 wt% rice husk flour • 3 wt% MMT • 0–8 wt% MAPE	Extrusion blown films	Composite films showed improved tensile strength and oxygen barrier properties	Majeed et al. (2014b)
Polypropylene (PP)				
PP	• 40–69 wt % wood dust • 2–6 wt % Cloisite®15A • 4 wt % MAPP	– Extrusion into a counter-rotating twin-screw extruder – Compression	– Low production costs – Limits the accumulation and disposal of wood industry waste	Madhoushi et al. (2014)
PP	• 0, 10, 20, 30 wt. % wood chips • 0, 1, 3, and 5 wt. % Cloisite 20A • 0, 3, 5, and 10 wt. % MAPP	Melt blending	– Improved thermal stability of composites – Increased tensile modulus	Tanasa et al. (2014)
PP	•10, 20, 30, 40, 50 wt%, respectively—wood flour treated with the dopamine hydrochloride • PP and MAH-g-PP (PP/MAH: E-43 Epolene) = 80/ 20)	Mixer mixing, secondly mixing into torque rheometer mixer, injection molding under pressure	The use of polydopamine-treated wood flour for preparing WPCs can be an efficient way to improve the water resistance of WPCs	Lanh and Thien (2016)
PP	• 0–60 wt% wood flour • 60 to 0 wt% charcoal powder • 3 wt% MAPP	Rotary drum blender, hot press molding	The swelling and water sorption considerably decreased along with the	Ayrilmis et al. (2015)

(continued)

Table 18.2 (continued)

Polymer matrix	Agro-industrial waste/additive	Manufacturing method	Results/ observation	References
			increasing charcoal content, while the strength and bending significantly improved	
PP	• 30 wt% biomass wastes (rice husk, coffee husk, coarse wool, landfill wood) • 24 wt% biochar • 4 wt% MAPP	Melt blending followed by injection molding	Wood and biochar composite exhibited the highest values of tensile/flexural properties with a relatively low peak heat release rate. Drastically reduced susceptibility toward fire	Das et al. (2018)
PP	• 40% filler corn stalk waste/flour • 6 wt% Epolene® G3015 as coupling agent (maleic anhydride-grafted polypropylene MAPP)	Mixing components in an internal mixer, palletizing, injection-molding	Tensile strength and modulus of the composites could be improved by using MAPP coupling	Flandez et al. (2012)
PP	• 0, 10, 20, 30, 40% corn cob (CC) • 3 wt.% MAPP		Tensile strength and elongation at break of biocomposites decreased with increasing corn cob loading, while modulus of elasticity increased	Salmah et al. (2014)
PP	•10, 30, and 50 wt % buckwheat husk • PP-g-MA	Injection molding	Mechanical properties do not differ significantly from those obtained with wood flour. Improved rheological characteristics	Andrzejewski et al. (2019a, b)

(continued)

Table 18.2 (continued)

Polymer matrix	Agro-industrial waste/additive	Manufacturing method	Results/ observation	References
PP	• 20/20 and 10/30% filler (talc/eggshell)	Blending with an internal mixer, palletized, pressed	The Young's modulus (*E*) improved with the increment of eggshell content	Toro et al. (2007)

additives. Since all components of the system are acting in synergy, the overall effect is a result of the combined effects of each of them interacting with the others.

18.3.2 Thermoset Polymer Matrix Biocomposites

As polymer matrices, thermosets, used alone or blended with other materials, undergo a cross-linking reaction during the curing process to form irreversible chemical bonds. Thermoset plastics always remain in a permanent solid state after an initial heat treatment, and therefore they are no longer affected by additional heat exposure. As a consequence, they cannot be melted, reshaped, recycled, and re-molded as part of a conventional recycling process. Thermoset plastics significantly improve the chemical and heat resistance, structural integrity, and mechanical properties like strength, hardness, and moldability of the resulted materials. Thermoset components that require tight tolerances, superior strength-to-weight ratio and performance, outstanding dielectric strength, low thermal conductivity, and resistance to corrosion are extensively used in high-heat applications such as electronics, appliances, automotive, electrical, lighting, and energy markets, and they are often used for sealed products due to their resistance to deformation. At the same time, thermosets present certain disadvantages such as low initial viscosity of materials which leads to supplementary operations, low tensile strength and ductility that need designs with thick walls, significant variation and less consistency from lot-to-lot in batch processes, poor thermal conductivity for housing replacements, and high rigidity resulting in product failure when used in high vibration applications (Väisänen et al. 2016).

Based on the two thermoset molding compound processes, namely reaction injection molding (RIM) and resin transfer molding (RTM), there are specific types of thermoset polymers, such as polydicyclopentadiene, polyurethanes and polyureas (RIM), polyesters, polyimides and vinyl ester (RTM), bismaleimide, epoxy, fluoropolymers, melamine, phenolics, polytetrafluoroethylene, polyvinylidene fluoride, silicone, and urea-formaldehyde. Thermoset composites consist in a large variety of formulations. Usually, matrix is a thermosetting resin (unsaturated polyesters, epoxies, vinyl esters, polyurethanes, etc.) that binds the reinforcement/filler (particles, chips, short or continuous fibers) together for an efficient transfer of load between them. Some properties of typical thermosets have been already illustrated in Table 18.1. The basic fabrication methods to prepare

Table 18.3 Thermoplastic composites with PS matrices

Polymer matrix	Agro-industrial waste/additive	Manufacturing method	Results/observation	References
Expanded PS waste	• Extruded PS waste • 10, 20, 30, and 40 wt.% of wood flour • 2 wt% coupling agent—a poly (styrene-co-maleic anhydride) oligomer with 30 wt.% of maleic anhydride groups	Processed in a co-rotating twin-screw extruder	The use of poly (styrene-co-maleic anhydride) as a coupling agent improved the compatibility between the wood flour and polystyrene matrix and the mechanical properties subsequently improved. The mechanical properties decreased as the wood flour loading increased.	Poletto et al. (2011)
Recycled PS	• Recycled PS • 20 wt% wood flour • 2 wt% three different styrene-co-maleic anhydride (SMA) oligomers as coupling agents	Processed in a co-rotating twin-screw extruder	The compatibilizers increased composite thermal stability and activation energy of composites.	Poletto et al. (2012)
Expanded PS waste	• 80 and 40 wt% expanded PS waste • 20 and 40% wt% wood flour • 2 wt % poly (styrene-co-maleic anhydride) as compatibilizer	Processed in a twin-screw co-rotating extruder	The mechanical and dynamic mechanical properties decreased with the wood flour loading, for composites without coupling agent. When the coupling agent was used, all these properties increased.	Poletto (2017)
Expanded PS waste	• Expanded PS waste • 20 w/w% of wood flour, with and without 1, 2, and 4 wt% of expanded PS • Styrene maleic anhydride as coupling agents, with different content of maleic anhydride groups, 30%, 25%, and 20% (w/w) and levels of 1, 2, and 4% in composites	Processed on a twin-screw co-rotating extruder	The addition of styrene maleic anhydride increased the wood plastic composites mechanical properties. The improvement in composite mechanical properties using SMA as coupling agent was strongly dependent on the amount of maleic anhydride.	Poletto (2016)
Expanded PS	• Expanded PS • 60% hemp shive particles	Preformed by cold mold pressure	The thermoforming pressure improved the mechanical properties of the composites.	Almusawi et al. (2017)

Table 18.4 Thermoplastic composites with PVC matrices

Polymer matrix	Agro-industrial waste/additive	Manufacturing method	Results/observation	References
PVC	• 0, 10, 20, 30, and 40 wt % rice husk powder • MA as compatibilizer	Mixed in a mixer grinder followed by compression molding	Mechanical properties of composites increased along with the amount of filler, while arc resistance decreased. Thermal and mechanical properties improved even more upon addition of MA.	Chand and Jhod (2008)
Un-plasticized PVC (PVC-U)	• 10–40 phr rice husk • Acrylic impact modifiers	Using a two-roll mill at temperature of 165 °C	Flexural strength for both unmodified and modified PVC-U composites increased up to RH loading of 20 phr. The tensile and impact strength of PVC-U composite decreased with RH loading.	Ahmad et al. (2010)
Plasticized polyvinyl chloride (PPVC)	• 0, 20, 30, and 40% rice husk	Molding on a hot press	Composites with 20% rice husk loading had the best mechanical properties compared to 30 and 40% rice husk loading due to high tensile and flexural strength.	Rahman et al. (2014)
PVC	• 16.7, 28.6, and 37.5 wt% wood sawdust	Extrusion	Tensile strength decreased with increasing moisture content up to 1–2% and then unexpectedly increased at higher moisture contents. Impact strength of the composites was considerably improved with	Sombatsompop and Chaochanchaikul (2004)

(continued)

Table 18.4 (continued)

Polymer matrix	Agro-industrial waste/ additive	Manufacturing method	Results/observation	References
			moisture content at low sawdust contents (16.7 wt%) and was independent of the moisture content at higher sawdust contents (28.6 and 37.5 wt%). A decrease in decomposition temperature with an increase in polyene content was evidenced with increasing moisture content.	
PVC	• 25 and 50% bamboo (*Bambusa vulgaris*) particle • Lubricant melting as additives	Blending with hot-cool mixing, granulation *via* counter-rotating extrusion, consolidation by compression molding	It was found that the thermal stability of composites depended on the particle size/ loading and the amount of lubricant used for processing. The oxidative induction time revealed that processing with bamboo particles does not contribute to degradation of PVC composites	Bahari et al. (2017)

thermoset composites include injection molding, compression molding, liquid molding (RTM), hand lay-up and spraying, filament winding, and pultrusion (Yashas Gowda et al. 2018). The manufacturing of thermosetting composites requires the mixing of dispersed phase and matrix at high temperatures, above the reaction temperature of the polymeric matrix.

18.3.2.1 Epoxy Resin-Based Biocomposites

Epoxy polymers, used as matrix resins for composites, have applications in electronics, aerospace, adhesives, insulators, and metal coatings owing to their desirable

Table 18.5 Thermoplastic composites with PA matrices

Polymer matrix	Agro-industrial waste/additive	Manufacturing method	Results/ observation	References
PA 6, PA 6,6 (Technyl A302 V25), PA 6,6 (Technyl A502)	– 20 and 30% flax (*Linum usitatissimum*) and jute (*Corchorus capsularis*)	Mixing in an internal mixer with two Banbury-like rotors, then injection molded	– Natural fibers were effective in reinforcing high-performance plastics such as polyamide 6 and polyamide 6,6 – An increase in both strength and modulus has been observed for all the fibers with respect to the unreinforced matrices	de Arcaya et al. (2009)
PA 6	– Flax fiber fabric and Kraft pulp cellulose – C20, a bifunctional activator – Aminopropyl-triethoxysilane (APS) and NaOH—for the surface modification of fibers	Anionic in situ ring-opening polymerization (ROP) using a special vacuum-assisted resin infusion process	– A pretreatment of the fibers was necessary in order to minimize the severe intrinsic inhibitive effect of cellulosic fibers on the ROP reaction – Optimum mechanical properties were obtained at a polymerization temperature of 150 °C	Kashani Rahimi and Otaigbe (2019)
PA 6	Recycled woven jute coffee bags (thickness of approx. 0.5 mm) coated with flexible epoxy resin	Compression molding	– The flexible epoxy coating (5 wt%) was able to improve the thermal degradation resistances – Flexural properties improved – SEM revealed strong fiber/matrix interfacial bonding for both coated and uncoated jute fabric composites	Thitithanasarn et al. (2012)
PA 6	– Up to 20 wt.% mixture of flax,	Melt compounding,	– Minor changes in the thermal	Kiziltas et al. (2016)

(continued)

Table 18.5 (continued)

Polymer matrix	Agro-industrial waste/additive	Manufacturing method	Results/ observation	References
	kenaf, and hemp fibers	followed by injection molding	properties of the composites – The storage modulus (e') increased as the natural fiber content increased	
Anionic polyamide-6 (APA-6)	• Textile-ramie fibers • Initiator—caprolactam magnesium bromide	Reactive processing	– Severe polymerization-inhibition and fiber-discoloration phenomena occurred – Initiator caprolactam magnesium bromide significantly suppressed inhibition and discoloration	Kan et al. (2015)
PA 6	– 30 wt. % raw and treated pineapple leaf fibers	Compounding by twin-screw extruder, followed by injection molding	– Treated fibers had superior interfacial adhesion over the untreated ones – Improved mechanical and thermal properties	Panyasart et al. (2014)
Co-polyamide 6/6.6/6.10	• 30–70 wt.% wood chips	Extrusion and hot pressing	– The tensile and bending strength increased by an increase in wood content, compared to wood-polyolefin composite materials – The thermal stability of composites was superior to that of wood	Zanoaga et al. (2004)

properties including superior thermal stability, low shrinkage, high stiffness, and excellent chemical resistance to environmental degradation. The poor mechanical properties and fragility of the epoxy matrix, as well as longer cure schedules and high monomer cost, have restricted its use in the automotive industry (Mittal et al.

Table 18.6 Thermoplastic composites with polymer blends matrices

Polymer matrix	Agro-industrial waste/additive	Manufacturing method	Results/observation	References
Recycled • HDPE (rHDPE) • Poly-ethylene terephthalate (rPET)	• 75:25% rHDPE/rPET • 40, 50, 60, 70, and 80 wt.% rice husk • MAPE as a coupling agent • EGMA as a compatibilizer	Extrusion process and compression molded	Mechanical properties significantly improved with the increasing amount of filler.	Ghani et al. (2014)
• Recycled polyethylene (rPE) • Recycled copolyamide 6/6.6/6.10 (r-coPA)	• 3–70% wood chips	Extrusion and compression molding	SEM confirmed the close contact between the wood and polymer matrix at their interface, which was evidence of good polymer–wood adhesion interactions.	Mamunya et al. (2006)
• Recycled polyethylene (rPE) • Recycled copolyamide 6/6.6/6.10 (r-coPA)	• 60% wood chips	Extrusion and compression molding	Improved bending strength, a better dispersion of the wood chips in matrix.	Zanoaga et al. (2016)
• PVC • Thermoplastic polyurethane (TPU)	• 20, 30, and 40 wt.% kenaf (*Hibiscus cannabinus*) bast fiber	Melt mixing	– Lower tensile strength and strain, and impact strength along with increased fiber content – Tensile modulus increased with the increased fiber content – High-impact strength was observed for 40% fiber content	El-Shekeil et al. (2014)

2018). Epoxy is a thermoset typically produced by reacting epichlorohydrin and a bis(epoxide) monomer, such as diglycidyl ether bisphenol A, with hardeners like anhydrides or amines, usually triethylenetetramine (Rahimi and Garcia 2017). Advanced composite materials with unique properties, cost effectiveness, and environment-friendly nature have been prepared using agro-industrial waste as reinforcement in epoxy matrix as an alternative to wood- and plastic-based components. Introducing coir pith and rice husk hybrid particulates obtained from waste agricultural residues into epoxy matrix produced customer value-added products (Prithivirajan et al. 2015). These composites, prepared by compression molding technique and particulate weight content ranging from 10 to 50%, exhibited considerably increased mechanical properties due to the attractive physical and mechanical properties of bioparticulates.

The microstructural, physical, mechanical, and thermal properties of epoxy polymer composites in the presence of oil palm boiler ash (OPBA) as reinforcement were studied using different filler loadings (10–50 wt.%) and different particle sizes (50–150μm) (Rizal et al. 2020). The experimental results showed that the incorporation of OPBA (53 wt.% silica content) in composites improved the physical, mechanical, and thermal properties of the epoxy matrix with maximum values recorded at 30 wt.% loading and size of 50μm. The utilization of this agro-industrial waste as filler in the structure of the composite material would benefit the palm oil industry by reducing waste load dispose to landfills and solid waste disposal cost and enhancing sustainability.

Particles of wood waste of *Pinus* sp. and teak (*Tectona grandis*) species as particleboard reinforcement phase and residual ink resin-based epoxy were applied to produce and to add value to new composites. The companies can use these panels for their economic and environmental advantages instead of disposing them off (Souza et al. 2018). Epoxy-based ink-waste melts at elevated temperatures and works as a possible binder in the consolidation of wood panels. As a consequence, these epoxy resins can be used as appropriate adhesives to reduce the demand of formaldehyde-based resins (urea-formaldehyde, melamine-formaldehyde, etc.) resulting in positive environmental and social aspects by decreasing in air releases of free formaldehyde in the production of panels. Economic benefits, especially for suppliers of wood waste regarding energy consumption for processing wood, can be gained in the production of panels from particles of wood waste of *Pinus* sp. and teak (*Tectona grandis*) species. Parameters, such as low-, medium-, and high-density panel classifications; epoxy resin content (20, 30, and 40%); and mixture of *Pinus* and teak wood waste particles (0, 25, 50, 75, 100%) were varied to manufacture and test the resulted particleboards. High density (up to 0.80 g/cm^3), 30–40% epoxy-based ink-waste adhesive, and 50–100% teak wood particles promoted a satisfactory performance in terms of physical and mechanical properties.

18.3.2.2 Polyurethane-Based Biocomposites

Among various applications, such as fibers and fabrics, adhesives, and sealants, rigid and flexible foams, coatings, elastomers, and polyurethanes are also used as polymer matrix in composites. Polyurethane-based composites are widely used as components, such as caravans, bumpers, covers, flaps, rear ends, hoods, and roof modules for commercial vehicles in automotive engineering due to their low-density, superior mechanical properties, and easy processability (Abdel-Hamid et al. 2019). Polyurethane composites present superior properties such as abrasion resistance, damping ability, high flexibility, and high elongation at breaking point, good weathering resistance and weathering durability, anti-aging, high impact strength, biostability, and low-temperature flexibility. Their applications are restricted by some drawbacks, such as high flammability, poor adhesion to metal surfaces, poor electrical and thermal conductivity, poor tensile strength, and thermal stability. The composites technology, in which particulate fillers, such as nanoparticles and natural fibers, are added to the polymers, may provide a good method to produce polyurethane hybrid nanocomposites and to solve the above-mentioned problems (Soundhar et al. 2019).

Polyurethanes are synthetic resins or plastics mainly synthesized by a polyaddition reaction between polyols and polyisocyanates. Vegetable oils such as soybean, palm oil, linseed oil, sunflower oil, and castor oil are renewable materials intensively employed in formulating polyurethane thermosets due to their good availability and high degree of functionalization possibility in manufacturing (Furtwengler and Avérous 2018). Several studies reporting the use of polyurethane resin as a binder for particleboards proved that the resulted materials meet the requirements of the international standards for physical and mechanical properties (Gava et al. 2015; Battistelle et al. 2016; Garzón et al. 2016; Varanda et al. 2018). The production technology of particleboards from alternative raw materials emerged as an interesting strategy to replace wood and dampen the deforestation. Introducing lignocellulosic wastes, such as corn cob (Akinyemi et al. 2016), flax and hemp (Sam-Brew and Smith 2015), topinambour and cup-plant (Klímek et al. 2016), poppy husks (Keskin et al. 2015), sorghum (Khazaeian et al. 2015), coconut and sugarcane bagasse (Fiorelli et al. 2018), and bamboo and sugarcane bagasse (Nakanishi et al. 2018) to produce particleboards could be an economic, social, and environmental strategy. These panels are typically manufactured from wood particles bonded by an adhesive or other synthetic binder, and the resulted aggregation is hot pressed till the curing of the resin.

The physical, mechanical, thermal, and aging properties of multilayer particleboards produced with green coconut (one inner layer, IL) and sugarcane bagasse fibers (two outer layers, OL) bonded with castor oil polyurethane resin were assessed (Fiorelli et al. 2019). The polyurethane resin was the bi-component castor oil polyurethane (50% polyol and 50% isocyanate), and its amount (by mass) for the OL and IL was 15% and 12%, respectively. Variations in the density profile across the thickness of the particleboards were evidenced by X-ray densitometry. Smaller fiber size and higher resin content led to a densification of the OL of the particleboards. All the particleboards survived a fatigue life of 40,000 cycles at stress levels equal to 25% of the breaking load as the fatigue tests indicated. Degradation of the particleboards under accelerated aging test indicated that the exposure conditions (temperature, humidity, UV irradiation) caused permanent damage to the physical (water absorption) and mechanical (tensile strength) properties of the materials.

Particleboards produced from sugarcane bagasse (*Saccharum officinarum*), jute (*Corchorus capsularis*), and curaua (*Ananas erectifolius*) fibers and their thermal, physical, and mechanical properties and microstructural characteristics were investigated (Fiorelli et al. 2018). The resin was the bi-component castor oil polyurethane adhesive prepared from 50% polyol and 50% isocyanate. By comparison with conventional wood particleboards, similar or superior physical and mechanical properties were observed when sugarcane bagasse (OL) and jute and curaua (IL) vegetable fibers of lower density were used as reinforcements for the multilayer particleboards.

The particleboards using vegetable fibers had higher values of their mechanical properties than those of the panels using only sugarcane bagasse with the same density, but the presence of the vegetable fiber did not influence the thermal conductivity. Addition of jute and curaua fibers induced higher porosity in the IL panels due to the geometry of the particle with a high value of aspect ratio (L/D). Although a higher porosity contributes to higher water absorption and lower values of internal bond, introducing jute and curaua fibers did not weak the particleboards. Particleboards containing only sugarcane bagasse and only jute fibers presented no significant variations in their physical and mechanical properties when subjected to accelerated aging tests.

Balsa wood (*Ochroma pyramidale*) waste and castor oil polyurethane resin in 11 and 15% content were used in the production of oriented strand boards (OSB) with different densities (300 and 400 kg/m^3) and 10 mm thickness, and their thermo-physical-mechanical properties were evaluated (Barbirato et al. 2020). OSB panels agglomerated with 15% of castor oil polyurethane resin and a density of 400 kg/m^3 had better physical and mechanical properties compared to the other formulations having lower density or lower resin content. Concerning the thermal behavior, all the treatments proved their potential in applications that require insulating properties. Modular panels made of reforested wood (*Eucalyptus* sp.), sugarcane bagasse, and bicomponent polyurethane resin based on castor oil were used in lateral closing for construction of crowding pens and chutes for cattle handling facilities (de Lucca et al. 2015). These modular panels, evaluated by the soft body impact testing, had physical and mechanical properties demanded by the normative documents for structural use. As a consequence, they can be used in cattle handling facilities because they showed structural performance necessary to withstand impact loads demanded by their usage.

Medium-density particleboard (MDP) panels were produced from coconut husk and *Pinus oocarpa* wood, and their properties (internal bond, the modulus of rupture and elastic modulus in static bending, moisture content, thickness swelling, and water absorption after water immersion for 2 and 24 h) were investigated (Narciso et al. 2020). *Pinus oocarpa* substituted with various content of coconut husk (25, 50, 75, and 100%), and 11% and 7% urea-formaldehyde adhesive (based on the weight of the particles) on the faces and in the core, respectively, were used to produce panels with a nominal density of 650 kg/m^3 and relation face/core/face ratio of 20:60:20. Water absorption properties (screw holding and thickness swelling) increased after 2 and 24 h, and mechanical properties (modulus of rupture, modulus of elasticity in static bending and internal bond) decreased by increasing the amount of coconut husk in the MDP panels. These experimental results met the values determined by standards, being allowed to state that it was possible to produce MDP panels only with coconut fiber.

New particleboards were produced from sugarcane bagasse in two different fiber lengths (5 and 8 mm) of the same density (1.0 g/cm^3) as a raw material and castor oil-based two-component polyurethane adhesive (Fiorelli et al. 2016). Their properties (density, thickness swell, absorption, modulus of elasticity, modulus of rupture in static bending and internal bond) were evaluated. Based on the fiber length, a significant difference between particleboards was revealed. The spaces between particles are filled with polyurethane adhesive, as evidenced by scanning electron microscopy, which contributed to improve the physical and mechanical properties of these particleboards. Waterproofed particleboards could be used in moist environments, as proved by a durability assessment based on accelerated aging tests.

Particleboards using industrial sawmill waste from tropical hardwoods (a mix of species) and sugarcane bagasse with castor oil-based bicomponent polyurethane resin were fabricated, and the influence of the incorporation of sugarcane bagasse (0, 10, 20, 30, 40, and 50%) on the physical and mechanical properties of the composites was evaluated (Yano et al. 2020). Some of the particleboards met standardized requirements, and the formulation 50% sawdust and 50% bagasse showed better performance, indicating the possibility for indoor use in dry conditions. The addition of sugarcane bagasse increased dimensional stability of particleboards when compared with panels manufactured with timber residue.

18.3.2.3 Amino Resin-Based Biocomposites

Amino resins are thermosetting polymers largely used in the furniture and construction industry; in coatings, laminates, and molding compounds; and in the production of wood-based panels, such as particleboards, fiberboards, and plywood. Urea-formaldehyde (UF), melamine-formaldehyde (MF), and melamine-urea-formaldehyde (MUF) are the main commercially used amino resins. Some other formaldehyde derivatives, such as phenol-formaldehyde (PF), tannin-formaldehyde (TF) and phenol-resorcinol-formaldehyde (PRF), and polymeric diphenylmethane diisocyanate (pMDI) are also used in manufacturing wood-based panels (Silva et al. 2015).

Urea-formaldehyde resin is widely used in the composites industry, especially in the forest products industry in the production of particleboard, medium-density fiberboard, and hardwood plywood, due to its special advantages, such as hardness, low flammability, noticeable homogeneity, good thermal properties, absence of color in the cured polymer, and adaptability of the resin to a variety of curing conditions. The main disadvantage of UF resin is its hydrolytic degradation in the presence of moisture and/or acids, which can be overcome by introducing other components like melamine into the UF resin molecules. MUF resin can replace UF resin especially to improve the properties of wood-based panels under high humidity conditions, because of its lower contribution to photochemical oxidation, environment, and human toxicity. By increasing melamine content in the MUF resin, formaldehyde air emission decreases. Free formaldehyde emission, one of the key environmental problems of formaldehyde-based resins, takes place from residual formaldehyde in the resin, during resin manufacture (when formaldehyde is generated by condensation reaction), and by hydrolytic degradation of cured resin during wood panel production. Free formaldehyde emissions are toxic to human health and potentially carcinogenic. There is a global trend toward biodegradable, non-pollutant, and renewable products. Therefore, numerous studies on finding new products to replace the above adhesives, such as castor oil-based adhesive, have been reported.

Waste biomass obtained after orchard pruning (plum and apple tree branches) was used to produce three-layered composite materials consisting in wood-chips bonded by UF resin (Kowaluk et al. 2020). The physical (thickness swelling, water absorption) and mechanical (internal bonding strength, screw withdrawal resistance, density profile and machining properties during milling and drilling) properties of the resulted particleboards were investigated. Modulus of rupture and modulus of elasticity fulfill the mechanical requirements for panels of furniture production and interior equipment purposes. A good performance of the composite materials elaborated from waste biomass expressed by the experimental results offers a potential revalorization of the two undervalued by-products.

A core layer made of mixture sugar beet pulp (SBP) and industrial wood particles aggregated with (UF) resin and a hardener (a 10% water solution of NH_4Cl) were used for production of three-layer particleboards (Borysiuk et al. 2019). Physical (density and density profile, thickness swelling, water absorption) and mechanical (modulus of rupture, modulus of elasticity, internal bond, screw holding) properties of the boards were studied. Investigation of the three-layer particleboards containing various proportions of SBP (10, 20, 30, 50, and 100%) in the core glued with UF resin showed that an increase in SBP content resulted in a decrease of modulus of rupture, modulus of elasticity, and screw holding and preserved the internal bond. An increase in SBP content in the core modified the density profile alignment and increased absorption and swelling of boards. Boards with 30% SBP met the requirements for furniture boards used in dry conditions.

The possibility of using agricultural residues (hazelnut husk, peanut hull, cotton stalk, corn stalk and licorice root) by using UF adhesives in manufacturing three-layer particleboards was investigated (Güler 2015). The UF resin was applied 8–9% for the core layer and 10–11% for the face layers based on oven dry weight. All the produced panels tested for mechanical (modulus of rupture, modulus of elasticity, and internal bond strength) properties complied with the minimum requirements in the standards for the general grade particleboards with the exception of thickness swelling and water uptake. Cup-plant (*Silphium perfoliatum* L.), sunflower (*Helianthus annuus* L.), and topinambour (*Helianthus tuberosus* L.) were used as raw materials for particleboards produced at a conventional density of 600 kg/m^3 (Klímek et al. 2016). Particleboards were glued with two different adhesives, pMDI and UF resins. It was shown that the obtained particleboards had acceptable performances, though some properties (modulus of rupture, thickness swelling, and water absorption) were below those obtained from conventional spruce particleboards. Even so, the requirements for general purpose particleboards used in dry conditions were met and the pMDI-bonded particleboards produced from agricultural residues were found to be a feasible alternative to conventional UF-bonded particleboards made from spruce.

The potential of poppy (*Papaver somniferum* Linnaeus) husk to manufacture wood-based particleboards was investigated, and the results claimed that it was possible to produce three layers of particleboards from poppy husk and pine wood using UF adhesive (Keskin et al. 2015). Particleboards were produced using poppy husk (up to 25%), 8% of UF resin for the core layer, and 10% for the outer layer, and 1% ammonium chloride was added to harden the adhesive. The physical and mechanical properties, formaldehyde emission, and limitation of oxygen index analysis met the requirements for interior fitments to use under dry conditions in the normative documents (EN 312). Formaldehyde emission is reduced and the feature of fire retardant for the manufactured particleboards is increased because of high limitation of oxygen index.

Miscanthus x giganteus stalks were studied as a possible replacement for wood in particleboards using 4 and 6% methylene diphenyl diioscyanate resination (Klímek et al. 2018). Modulus of rupture, modulus of elasticity, and internal bonding strength had lower values than those of spruce-made particleboards. Particleboards from *Miscanthus* showed collapsed cell regions in the soft parenchyma, with no obvious adhesive failures after internal bonding testing, suggesting a connection with the reduced mechanical properties. In contrast, particleboards made with spruce revealed much smoother fracture surfaces with structural failures running through cell walls and possibly also through gluelines. The *Miscanthus*-made particleboards exhibited higher thickness swelling, but lower water absorption in comparison with spruce-made particleboards. For *Miscanthus*, no effects of higher pMDI adhesive dosages on modulus of rupture, modulus of elasticity, and internal bonding strength were observed.

The efficiency of some particleboards manufactured using particles from sugarcane bagasse and eucalyptus residues of the pulp industry was investigated using castor oil-based polyurethane resin (PU-Castor) and UF resin (Sugahara et al. 2019).

When 60% wood particles and 40% bagasse and a 10% adhesive dose based on the dry mass of particles composed the particleboards, their physical (apparent density, moisture content, thickness swelling after 24 h) and mechanical (modulus of rupture, modulus of elasticity to static bending, and internal bond strength) properties were investigated. The experimental results proved the potential of eucalyptus and sugarcane bagasse residues in the production of high-density particleboards. Greater efficiency showed the panels produced with PU-Castor, and their physical and mechanical properties met the requirements necessary for their use in dry conditions.

Rice husk without any further treatment was processed into particleboards using PF resin as binder (Ciannamea et al. 2017). Processing parameters, pressure and binder content on the density, water absorption, thickness swelling, modulus of rupture, and modulus of elasticity were investigated. Particleboards with 11% of binder content met the minimum requirements of modulus of rupture and modulus of elasticity recommended for commercial use, while particleboards with 14% binder content also accomplished the requirements for industrial use, finding a resourceful use for this agricultural waste.

18.4 Multicomponent Polymer Systems Comprising Biopolymers from Renewable Resources and Agro-Industrial Waste

The society generates large amount of waste—both non-degradable (e.g., petroleum-derived) and degradable (e.g., from agriculture and food industry). A sustainable approach to decrease the resulted amount of waste may include all aspects related to the manufacture of bio-derived products from producing chemicals through complex biomass capitalization to introducing feasible polymerization strategies and further assembly into bioplastics with environmental friendliness and whole recyclability features. Up-to-date information (De Corato et al. 2018) emphasized that the issues of utilization and application of integrated biorefinery from remaining parts resulted from agricultural and industrial processes and agro-waste are somewhat underrepresented and they require additional attention in the near future. Among renewable biomass, the first remaining parts are produced in the plains, while the indirect wastes resulted from crop processing. Such agricultural residues, as well as food waste, may be transformed for varied and valuable chemical production by implementing suitable biorefining strategies (De Corato et al. 2018; Pleissner et al. 2017).

A durable progress in society is also attained through designing eco-friendly and versatile plastic materials. Furthermore, it is essential that such materials be recyclable to a large extent, being produced based on a lasting use of the both fossil and green carbon sources. For a long time, the plastic materials derived from petroleum and bio-derived sources will coexist and bring an outstanding contribution in the creation of viable and competitive products, thus fulfilling the increasing demands in the contemporary communities (Mülhaupt 2013) where polymer materials are designed in order to exhibit stiffness, softness, rubber behavior, conductance, or insulation properties, as well as transparency or opacity, permeability or impermeability, and stability or biodegradability. The significant potential afforded by the polymers derived from agricultural sources mainly those that are biodegradable like starch was from a long time established (Averous and Halley 2009). The influence of sustainable starch materials on the macro- or nano- structural level and further processing, thermo-mechanical behavior, and efficiency of plasticized starch-based materials were previously presented in a comprehensive review (Averous and Halley 2009).

Polymers from agro-resources are obtained mostly from plant species and have compostable and renewability features, at the same time manifesting some ordinary behavior, such as the high water affinity. Majority of such polymers can be engineered by a direct route as fillers, either through plasticization or chemical modification. Accurately, polyoses, proteic compounds (e.g., gluten or zein), and aromatic polyphenolic polymer, namely, lignin, are such polymers. The available in large quantities class of polymers from agro-resources is represented by polyoses, with various provenience sources and chemical structural features, such as cellulose, chitin, and starch. Biopolymers and some of their applications are schematically presented in Fig. 18.3. Some biopolymers, namely, starch, PLA, and lignin, used as

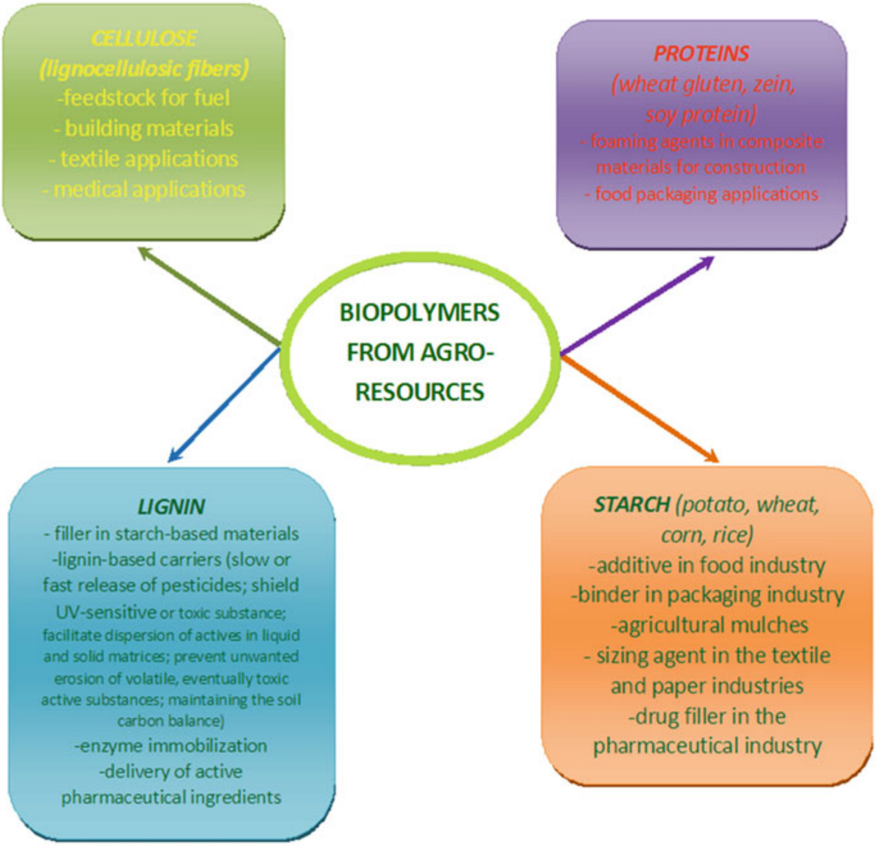

Fig. 18.3 Biopolymers from agro-industrial resources and some of their applications

matrix in composites with agro-industrial waste reinforcements will be presented in the following sections.

18.4.1 Starch

Starch, a relatively low-cost polyose obtained from renewable sources, was studied to a large extent to be used as an effective alternative for plastic materials derived from petroleum sources mainly as thermoplastic starch (TPS) which results under high temperature and shear conditions (Averous and Halley 2009; Pushpadass et al. 2009; Chang et al. 2010). Its chemical structure is formed by amylase, a linear component, and amylopectin, a highly branched component, with crystalline and amorphous regions in alternating layers, their ratio significantly influencing the properties of the resulted bio-based materials. Due to their better properties (increased thermal stability, superior mechanical strength, reduced water affinity), the amylose-rich materials are usually preferred than amylopectin-rich materials. There was a previous study (Pang et al. 2014) which investigated the interesting performance behavior of the thermoplastic starch/polyolefin mixtures where starch is derived from agricultural waste presenting different ratios for the structural components (amylose/amylopectin), the amylose content being lower than in a normal pristine starch. The characteristics of starch, like high temperature value for glass transition, strong inter- and intra-molecular hydrogen bonding, reduced water resistance, and reduced flowing ability in granular form, make the processing methodology to be difficult which therefore determines a limited applications area. All these above-mentioned drawbacks can be solved through suitable approaches such as addition of plasticizers, blending with polymers and nanofillers, chemical modification, or an appropriate combination of the former listed (Mekonnen et al. 2013; Teacă and Bodîrlău 2015; Bodîrlău et al. 2014; Teacă et al. 2014; Xie et al. 2014).

Plasticizers like water, glycerol, sorbitol, fructose, mannose, glycol, and fatty acids (some of them even in combination like water and glycerol) are largely used for starch plasticization which is the most applied methodology for improving starch properties. In spite of the fact that the chemical modification can generate harmful by-products and implies relatively high costs, starch can be successfully modified for enhancing both its properties and polymer systems comprising the modified starch (Zamudio-Flores et al. 2010; Bodîrlău et al. 2012). The use of waste derived from agriculture practices as a source of starch for production of bioplastics may be advantageous for local farmers which can valorize such waste not only as nutrients for animal feeding. Furthermore, the lower cost of such agricultural waste enriched in starch is more conveniently in comparison with that of native sources of starch. In this context, significant starch amounts can be found in maize (corn), rice, and wheat, alongside with other sources (Gupta 2011) such as potatoes, sweet potatoes, cassava (known as yuca or manioc), banana (mainly peels), barley, buckwheat, rye, and taro (*Colocasia esculenta*, a tropical Asian plant grown primarily as a vegetable food for its edible corms which are very rich in starch). Another important source of starch is represented by sugar palm (*Arenga pinnata*) which alongside its fibers is largely considered as a significant agro-industrial waste in the agricultural industry from tropical Asian countries with potential use for biocomposites production (Sahari et al. 2013; Sanyang et al. 2016, 2017; Edhirej et al. 2017a, b; Ibrahim et al. 2020). The sugar palm tree can be an attractive source for a starch suitable for plasticization with beneficial effects upon properties of materials resulted through blending it with other polymers or fibers. The fibers extracted from the sugar palm trees contain a significant amount of cellulose as major chemical polymer component (66.49%) which confers them significant mechanical properties (Sanyang et al. 2016). In Table 18.7, some examples of applications of starch in multicomponent polymer systems comprising agro-industrial waste, where it is usually present as thermoplastic polymer matrix, are presented.

Table 18.7 Use of starch in multicomponent polymer systems comprising agro-industrial waste

Type of starch	Type of agro-industrial waste	Type of polymer system and processing	References
Corn starch	By-products from cassava and ahipa starch extraction (cassava and ahipa peels and bagasse)	Corn starch plasticized using glycerol and mixed with agro-residues; thermo-compression	Versino et al. (2020)
Tapioca starch	Water hyacinth fibers (WHF)	Tapioca starch plasticized using glycerol and mixed with WHF's water solution Solution casting method	Abral et al. (2018), Asrofi et al. (2018)
Corn starch	Cornstalk Fiber (csf)	Corn starch thermoplasticized using fructose as plasticizer; solution casting and dehydration technique	Ibrahim et al. (2019)
Corn starch	Corn stalk Sugar palm fibers (spf)	Corn stalk/sugar palm fiber reinforced corn starch biopolymer hybrid composites Solution casting method	Ibrahim et al. (2020)
Sugar palm starch	Sugar palm fibers (SPF) *Eucheuma cottonii* seaweed waste	Seaweed/SPF reinforced Sugar palm starch/agar bio-hybrid composite; using glycerol as plasticizer; melt-mixing and hot pressing	Jumaidin et al. (2017a)
Sugar palm starch	*Eucheuma cottonii* seaweed waste	Thermoplastic sugar palm starch/agar (TPSA) blend containing seaweed waste as biofiller; using glycerol as plasticizer; melt-mixing and hot pressing	Jumaidin et al. (2017b)
Sugar palm starch	Woody fibers from sugar palm tree for starch extraction	Thermoplastic sugar palm starch/agar blends; using glycerol as plasticizer; melt-mixing and hot pressing	Jumaidin et al. (2016)
Commercial thermoplastic starch	Sugarcane bagasse fibers	Starch-based biopolymer reinforced with sugarcane bagasse fibers	Jiménez et al. (2016)
Sugar palm starch	Sugar palm fibers from which nanocrystalline cellulose was extracted using delignification and mercerization	Sugar palm nanocrystalline cellulose reinforced sugar palm starch bionanocomposites; sorbitol and glycerol as plasticizers; solution casting method	Ilyas et al. (2018)
Corn starch	Blueberry agro-industrial waste	Thermoplastic corn starch/ blueberry powder films; using glycerol as plasticizer Solution casting method	Luchese et al. (2017)

(continued)

Table 18.7 (continued)

Type of starch	Type of agro-industrial waste	Type of polymer system and processing	References
Cassava starch	Blueberry agro-industrial waste as pomace in powder form	Cassava starch films incorporated with blueberry pomace; using sorbitol as plasticizer; solution casting method	Luchese et al. (2018)
Corn starch	Oil palm frond (OPF) fiber	Oil palm fiber-reinforced thermoplastic starch composites; OPF pretreatment with ionic liquids; corn starch plasticization using glycerol as plasticizer; compression molding technique	Mahmood et al. (2017a, b)
Native cassava starch from cassava tubers	Sugar palm fibers	Sugar palm fibers reinforced thermoplastic cassava starch films; using fructose as plasticizer; solution casting method	Edhirej et al. (2017a, b)

18.4.2 Polylactic Acid (PLA)

*Polylactic acid (PLA)*is an aliphatic-type thermoplastic polyester, biodegradable, and recyclable, different than most thermoplastic polymers as it is derived from renewable resources, namely, agricultural residues, waste, and by-products like corn starch, tapioca (roots, chips or starch), or sugarcane (Mekonnen et al. 2013). From these sources, the lactic acid (LA) can be produced through suitable biotechnology routes as a platform chemical and precursor for production of biodegradable, biocompatible LA polymers (e.g., either by condensation of lactic acid or ring opening polymerization of lactide which is the diester of lactic acid). These polymers are very promising thermoplastic polymers mostly used for high-end applications (Drumright et al. 2000), ranging from technical textile fiber applications, furnishing, and agricultural products to pharmaceuticals and drug release systems as well as medical textile industry, namely, also biotextile, which include suture materials, implantable matrices, and organ reconstruction (Gupta et al. 2007). The sustainable use of polylactic acid in these applications is based not only on its biodegradability and its provenience from renewable resources but also on its ease processing and well behavior under technological conditions providing excellent properties with low expense.

A recent review (Djukić-Vuković et al. 2019) focused on the sustainable production of lactic acid using residues and wastes derived from agricultural practices through different methodologies considering all aspects involved. The characteristics of PLA-based polymer matrix can be accordingly modified through complex interrelations occurred when using varied fillers, both organic and inorganic type, including by-products and wastes. Reinforcement and toughness effects can be thus determined by the rigidity of used particles and the structural changes in PLA macromolecular chain (Battegazzore et al. 2014a, b, c; Barczewski et al. 2020). Another interesting way to modify and develop enhanced characteristics in PLA for its use as an alternative sustainable biopolymer in applicative end-uses including automotive and electronics ones is addition of nanofillers—nanofibers and nanoparticles (Raquez et al. 2013)—in order to overcome the inherent shortcomings of PLA (reduced thermal stability, decreased crystallization rate), while properties such as flame retardancy, conductance, resistance against UV radiation, and microorganisms and the barrier ones are essential for many other end-use areas. Some examples for use of PLA in multicomponent polymer systems comprising agro-industrial waste are presented in Table 18.8.

18.4.3 Lignin

Lignin represents an aromatic biopolymer of polyphenolic type, constituent of the lignocellulosic materials, which is responsible for the mechanical resistance of plants being intertwined between the polysaccharide components, namely, cellulose and hemicelluloses, conferring rigidity to the plant cell walls. Lignin has the most complex structure as derived from oxidative coupling of p-hydroxycinnamoyl alcohols (monolignols) and related compounds. The processes for assembling structural units and their activation toward lignin polymerization are under strict biological control; even these occur via simple combinatorial chemistry pathways (Tobimatsu and Schuetz 2019). Comparatively with the use of cellulose and hemicelluloses, lignin is still the most under-utilized of the biopolymers from lignocellulosic biomass despite its large potential for generation of renewable fuels and value-added chemical compounds in relation with many progresses made in research areas such as bioengineering, biorefinery, and catalysis (Rinaldi et al. 2016).

Actual approaches for introducing lignin in latest designed materials focus on its combination with other polymer matrices, either natural or synthetic, through appropriate covalent modification and blending strategies. The significance of lignin in technology applications and focused attention on its sustainable blending and copolymerization strategies for use in a large applicative area were previously reviewed (Chung and Washburn 2013). For example, the blending of two different kinds of biopolymers derived from renewable resources, lignin, a tough polymer matrix and biodegradable starch, has great potential when considering the major drawback of the starch which exhibits a pronounced hydrophilic behavior in various environmental conditions. In this context, lignin confers additional features to starch matrix when result films and foams with better properties thus becoming potentially attractive for a broad applicative area. As example, starch-lignin blends with enhanced properties were obtained through use of chemical modification of starch polymer matrix and adequately its appropriate plasticization strategy (Spiridon et al. 2011).

Table 18.8 Uses of PLA in multicomponent polymer system comprising agro-industrial waste

Types of polymer system based on PLA	Type of agro-industrial waste	Type of processing	References
Biocomposites	Hemp hurd (as chips and powder), alfalfa, and grape stem	Melt blending	Battegazzore et al. (2019)
Films	Water-soluble extracts from banana pseudo-stem as functional additives	Solution blending, casting, and thermo-compression method	Sango et al. (2019)
Biocomposites	Cellulose extracted from rice husk	Melt blending	Battegazzore et al. (2014d)
Fully bio-based and biodegradable composites	Almond shell flour	Maleinized linseed oil as a reactive compatibilizer during the extrusion process; melt compounding	Quiles-Carrillo et al. (2018)
Biocomposites	Bagasse agave tequilana fibers pretreated using alkali and enzymatic stages	Extrusion and press molding	Huerta-Cardoso et al. (2020)
Composites	Chestnut shell waste	Melt blending without hydrolytic degradation of the bio-polyester PLA Matrix	Barczewski et al. (2018)
Bio-nanocomposite films	Cellulose nanocrystals derived from coffee silverskin	Melt compounding: Extrusion and press molding	Sung et al. (2017)
Composites	Cork-wood hybrid Filler system	Melt compounding: Extrusion/injection molding process; evidencing the potential of using cork fillers to improve the dimension stability of composites in the presence of moisture	Andrzejewski et al. (2019a, b)
Composite films	Cellulose nanocrystals from waste cottonseed hulls	Solvent casting method; using maleic anhydride ma as both the compatilizer and crosslinker	Zhou et al. (2018)
Biocomposites	Algae waste (red, brown, and green) as filler	Melt mixing; algae can be used as filler in thermoplastic composites up to at least 40 wt% loading, since the stiffness of materials can be maintained	Bulota and Budtova (2015)
Composite films	Corn Stover organosolv lignin from biorefinery	Melt compounding: Extrusion and press molding	Gao (2017)
Composites	Coffee silverskin (CS) as natural filler being	Melt blending	Ghazvini (2018)

(continued)

Table 18.8 (continued)

Types of polymer system based on PLA	Type of agro-industrial waste	Type of processing	References
	by-product of coffee roasting process		
Biocomposites	Rice husk as source of silica	A semi-industrial process of extrusion	Battegazzore et al. (2014c)
Natural filler-based composites	Cellulose fibers, wood sawdust, hazelnut shells, flax fibers, corn cob, and starch from the Italian agricultural production	Melt blending	Battegazzore et al. (2014a)
Biocomposites	Natural additives from hazelnut skin and cocoa by-products through a selective and serial extraction to be used as plasticizers for PLA, antioxidants for PP, and reinforcement fillers for both polymers	Melt blending	Battegazzore et al. (2014b)

An effective valorization of lignin implies an attentive consideration of both multiple upstream (e.g., bioengineering approaches, extraction methodology, conversion by catalysis) and downstream (e.g., lignin depolymerization and upgrading) processing phases. Such strategy is a prerequisite for achievement of a sustainable economy and to reduce the carbon footprint. There are many potential uses of lignin for high-value products ranging from fuels and chemical platforms to filler materials, namely, plant-derived plastics and composites (engineering plastics and thermoplastic elastomers, polymeric foams and membranes), as well as low cost carbon fibers (Azadi et al. 2013; Ragauskas et al. 2014; Rinaldi et al. 2016; Becker and Wittmann 2019; Collins et al. 2019). Some examples for the use of lignin in multicomponent polymer systems comprising agro-industrial waste are illustrated in Table 18.9.

18.5 Conclusion and Future Trends

The use of composite materials based on agro-industrial waste has been expanding in the recent decades. This trend is encouraged by industry and agriculture, on one hand, because it helps reduce the demand of the fossil resources as raw materials, limits the waste disposal, and supports the recovery and re−/up-cycling of waste, contributing thus to the joint effort to protect the environment. On another hand, the properties of these composites are satisfactory, although not at the same level of performance as typical plastic composites, but their lightweight and low cost, in addition to their other advantages, make them highly attractive for the consumers. For example, composites panels made of polymer matrices and various types of

Table 18.9 Use of lignin in multicomponent polymer systems comprising agro-industrial waste

Type of polymer system based on lignin	Type of agro-industrial waste	Type of processing	References
Thermoplastic cassava starch-based composites	Kraft lignin and esterified lignin from	Mixing, compression molding	Kaewtatip and Thongmee (2013)
Chemically modified starch microparticles (using malic acid)/ glycerol plasticized corn starch/lignin filler composite films	Industrial Kraft lignin extracted by precipitation in the lignoboost process	Solution casting	Bodirlău et al. (2013)
Chemically modified starch microparticles (using adipic acid)/ glycerol plasticized corn starch/lignin filler composite films	Industrial Kraft lignin extracted by precipitation in the lignoboost process	Solution casting	Spiridon et al. (2011)
Blends with polylactic acid	Lignin extracted from almond shells by organosolv process and acetylated	Extrusion	Gordobil et al. (2014)
Lignin–formaldehyde matrices for biobased composites reinforced with sisal fibers	Lignin isolated through an industrial organosolv acid hydrolysis process from sugarcane bagasse (Dedini rapid hydrolysis DHR)	Mixing and compression molding (the presence of lignin in both fiber and matrix Greatly improved adhesion at the fiber–matrix interface)	Ramires et al. (2010)
Fiberboards	Sugarcane bagasse Lignophenolic prepolymers and fibers	Mixing, compression molding	Hoareau et al. (2006)

agro-industrial waste have been successfully employed in structural applications because they showed adequate mechanical strength and dimensional stability, good screw holding capacity, and low water sorption.

Agro-industrial wastes such as rice husk, bagasse, palm oil, wood chips and flour, cereal straws, eggshells and seashells, feathers, plant stalks, natural fibers, etc. are able to provide much higher strength per unit mass than some inorganic fillers (carbon black, calcium carbonate, talc, zinc oxide). They also content a high amount of silica which may have a negative effect on the processing equipment. Since agriculture waste depends on localization and season, agriculture practice and harvesting, transport, storage, and processing, it is easy to understand that their characteristics vary within large limits for the same species. In order to counterbalance these drawbacks, the other elements of the multicomponent systems must be wisely chosen: the nature and type of polymer matrix; type of pretreatment, functionalization method, or compatibilization strategy; number and nature of additives, in correlation with the application; processing methods and parameters; and type of equipment. As shown in this chapter, agro-industrial waste was successfully used as reinforcement in various polymer matrices: synthetic thermoplastics and thermosets, and biopolymers. The thermoset biocomposites have significantly improved chemical and thermal stability, structural integrity, mechanical properties (strength, hardness), and moldability, but they cannot be melted, reshaped, recycled, and re-molded in order to be introduced in a new production cycle. By comparison, the thermoplastic biocomposites showed an enhanced processability, increased elastic properties, and a high degree of post-consumer recovery, due to the low melting point of the polymer matrix. As for the composites based on biopolymers (starch, lignin, PLA) as matrix, they are promising materials due to their high added-value and biodegradable character, despite their low rate of recovery.

The literature of the recent decades is abundant in reports on multicomponent polymer materials based on agro-industrial waste, with an emphasis on the variety of formulations and procedures. Unfortunately, only a small amount of data is effectively transferred to industry and applied, although constant progress is evident and encouraged. The wide range of inexpensive and locally available raw materials represent a potential leverage that may help industrial and individual consumers to switch their preference from classic plastic materials to the new bio-based materials as they can provide promising benefits and contribute to a sustainable development. Constant concerns over the climate changes and global food security have been motivating the development of bio-based materials. This trend may be regarded as a challenge to both academic and industry R&D scientists and policy makers, because manufacture of bio-based materials, including the valorization of agro-industrial waste, requires land and crops which are used less for food.

Nevertheless, future research must focus on the development of new high added-value products and methods, and on up-grading the existing materials, in close relation with the local availability of materials and processing facilities. The design of new procedures or hybrid methods may be approached simultaneously with the use of the already existing ones in various combinations. Another very important aspect, which has been so often neglected, is the efficient, low-cost production without generating secondary pollution (waste that cannot be further recycled). Still, this field of research is very active, so it is reasonable to expect new and exciting developments.

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Polysaccharide-Based Materials as Promising Alternatives to Synthetic-Based Plastics for Food Packaging Applications

19

Irina Elena Raschip, Nicusor Fifere, and Maria Valentina Dinu

Abstract

Nowadays, the movement of goods without packaging is almost unthinkable; therefore, the implementation and development of new packaging products and processes are highly required. In designing two-component product-package systems, both the impact of the raw materials and their elimination or reuse after exceeding their lifetime must be taken into account. Consequently, abundant, nontoxic, renewable, biocompatible, and eco-friendly polymers like polysaccharides (chitosan, alginate, xanthan gum, dextran, salecan, pullulan, and curdlan) are good alternatives and key factors to reduce the quantity of plastics, which are practically nonbiodegradable. In addition, the antioxidant-loaded polysaccharide films or coatings have been shown to exhibit an important function in safeguarding of foods against bacterial spoilage organisms by their remarkable antioxidant activity. Anyway, the bio-based packaging materials cannot take fully the place of the synthetic-based plastics, but they can lower the total cost of packaging and the amount of the plastics exploited. Main factors which control the synthesis of polysaccharide-based packaging materials and their morphological, mechanical, thermal, and water sorption features are discussed in this chapter based on the recently published articles and our own results.

Keywords

Biodegradable · Polysaccharides · Biological films · Food packaging · Applications

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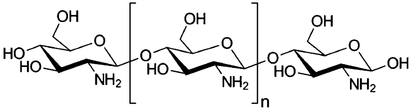
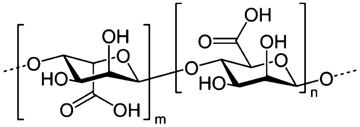
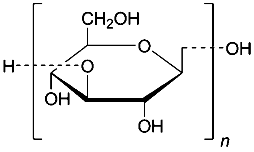
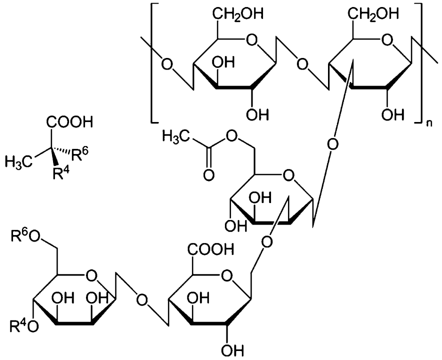
19.1 Introduction

The development of new advanced bioactive packaging materials, especially based on polymeric compounds, is a priority direction of modern chemistry aimed to improve the existing food technologies. Currently, food industry encounters numerous challenges; the reducing of the pollution caused by the use of plastics and their derivatives in the packaging of food with a short shelf life is a major one (Muscat et al. 2012). Consequently, in recent years, significant steps have been taken hold into developing new intelligent, eco-friendly packaging, having embedded biologically active compounds that can offer antimicrobial and antioxidant functionalities to the packaging materials (Raschip et al. 2020a, b, c). The packaging materials are intended to temporarily safeguard a product or a set of products against bacterial organisms attack and chemical or mechanical damage and thus to maintain their quality and integrity during handling, transport, and storage (Cazon et al. 2018). Therefore, the production of plastic-based food packaging has risen very rapidly and has led to serious environmental issues since synthetic plastics are resistant to degradation (Muscat et al. 2012). In this regard, a plethora of studies have been concentrated to find new alternatives for synthetic-based plastic films. A very promising one is that based on polysaccharides as raw biodegradable materials to improve food preservation and reduce the use of chemical additives (Persin et al. 2011; Tharanathan 2003; Cazon et al. 2017).

The good film-forming capacity of several polysaccharides (chitosan, alginate, xanthan, dextran, starch, curdlan, and pullulan) could lead to new opportunities to expand the field of the food packaging systems (Table 19.1; Cazon et al. 2017; Dutta et al. 2009; Espitia et al. 2014; Shankar et al. 2017). Polysaccharide-based films are mainly prepared by a facile and versatile technique, such as, solvent casting (Bajić et al. 2019). In most of the cases, the addition of plasticizers, nanofillers, and other natural or synthetic polymers is required to obtain polysaccharides-based films with improved/enhanced water vapor permeability (WVP), chain flexibility, as well as elongation at break (Raschip et al. 2020a; Muscat et al. 2012; Cazon et al. 2017, 2018; Dutta et al. 2009; Shankar et al. 2017; Zemljic et al. 2013; Narasagoudr et al. 2020; Han et al. 2018; Ho et al. 2020; Namratha et al. 2020; Surendhiran et al. 2019; Zhou et al. 2019; Sheet et al. 2018; Shao et al. 2018). The commonly added additives are surfactants, polyols, sugars, and lipids (Espitia et al. 2014).

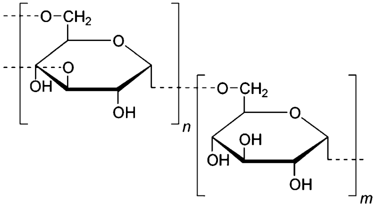
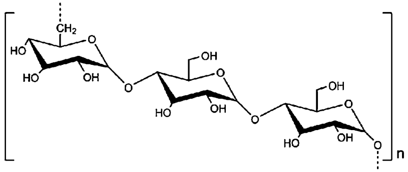
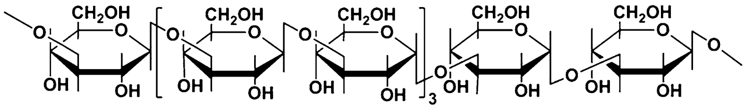
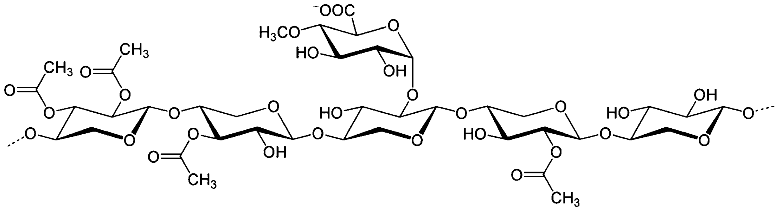
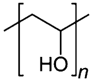
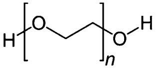
The polysaccharides-based films can act as bioactive food packaging by embedding of various agents able to enhance the antioxidant and/or antimicrobial features and therefore the foodstuff protection capacity against bacterial spoilage organisms (Bajić et al. 2019; Halász and Csóka 2018; Jing et al. 2019; Sogut and Seydim 2018; Sun et al. 2018; Biao et al. 2019; Kim et al. 2018b; Olivo et al. 2020; Shankar et al. 2019; Zare et al. 2019; Wang et al. 2020; Outukia et al. 2016; Raschip et al. 2020b, c; Sharma and Ramana Rao 2015; Riaz et al. 2018). This approach consists of entrapping antioxidant agents within the polysaccharide matrix which give rise to stable and protective packaging films for food products with sensitivity to oxidation. So far, numerous active compounds from plant, fruit, and seed extracts including tannic acid, gallic acid, ferulic acid, curcumin, anthocyanidins, quercetin,

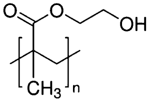
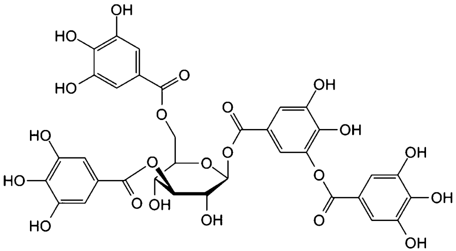
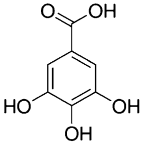
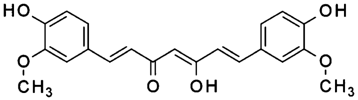
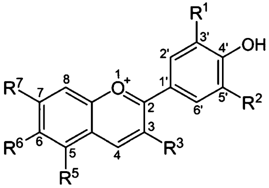
Table 19.1 Structure of some polysaccharides, synthetic polymers, and of several active compounds identified in fruits, vegetables, or plant extracts used to obtain new bioactive packaging materials

Polysaccharides	
Chitosan  	**Alginic acid** 
Curdlan  	**Xanthan** 

(continued)

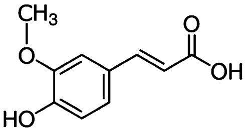
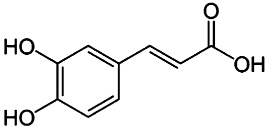
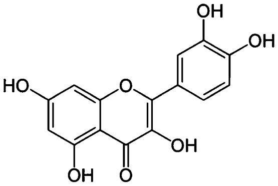
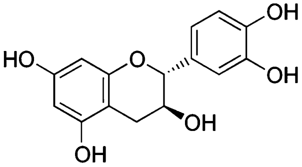
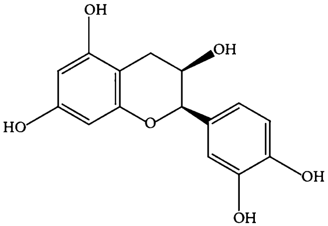
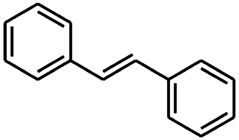
Table 19.1 (continued)

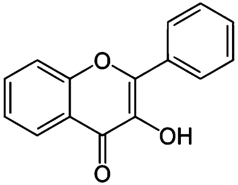
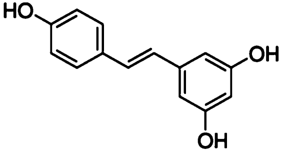
Dextran 	**Pullulan** 
Salecan 	
Xylan 	
Synthetic polymers blended with polysaccharides	
Polyvinyl alcohol 	**Polyethylene glycol** 

Poly(2-hydroxyethyl methacrylate) 	
Several of the active compounds identified in fruits, vegetables, and plants extracts	
Tannic acid 	**Gallic acid** 
Curcumin 	**Anthocyanidins** 

(continued)

Table 19.1 (continued)

Ferulic acid  	**Caffeic acid** 
Quercetin  	**Catechin** 
Epicatechin  	**Stilben** 

Flavonols 	**Resveratrol** 

epicatechin, catechin, resveratrol, and so on (Table 19.1) have been investigated in regard to their effects on the functional features of the packaging films stated for food protection against oxidation (Benedec et al. 2013; Mir et al. 2018).

Phenolic compounds, found in fruits or vegetables, are recognized as very efficient free radical scavengers belonging to the group of primary antioxidants (Benedec et al. 2013). They received considerable attention due to the fact that they possess remarkable biological activities (Xia et al. 2010). Some of the most recent studies on antioxidant-loaded polysaccharide-based films and coatings will be summarized in this chapter.

19.2 Polysaccharides for Preparation of Bio-Based Materials with Antimicrobial and Antioxidant Properties

19.2.1 Chitosan

Chitosan (CH), a cationic polysaccharide comprising of (1,4)-linked 2-amino-deoxy-D-glucan, is obtained by the partial hydrolysis of the *N*-acetyl groups of chitin. CH has been extensively investigated for environmental, biomedical, biotechnological, and food packaging applications in view of its remarkable features including nontoxicity, biodegradability, biocompatibility, chelating ability of various metal ions, as well as low permeability to oxygen, good film-forming property, and inherent antibacterial activity (Cazon et al. 2017; Dehghani et al. 2018; Dinu et al. 2013, 2018; Dutta et al. 2009). The pH values and the deacetylation degree (DD) are two crucial factors that affect the CH attachment to bacterial cell membrane (Aider 2010). Higher DD values ($DD > 80$) and acidic aqueous medium are responsible for the increase of the antimicrobial activity of CH. At low pH values (up to 5.5), the amino groups of CH are protonated and thus can easily interact with the negatively charged bacterial cell membranes inducing the cell death (Elsabee and Abdou 2013; Dutta et al. 2009). In food products, CH offers a wide range of functions (Miteluț et al. 2015), including safeguarding of food from microbial attack impeding the growth of not only gram-positive and gram-negative bacteria but also molds and yeasts (Rollini et al. 2017), enhancer of surimi and fishery products gelation, or deacidifier of the fruit juices (Zemljic et al. 2013). However, neat CH films have some major disadvantages such as poor barrier to moisture, brittleness, and weak mechanical properties (Bourbon et al. 2011; Silva-Pereira et al. 2015), which hamper its use for a wide area of applications. To improve these properties blending with other natural or synthetic polymers, like xylan (Kamdem et al. 2019; Vadivel et al. 2019), starch (Haria et al. 2018), gelatin (Shahbazi 2017), polyethylene glycol (PEG) (Bi et al. 2020), poly(2-hydroxyethyl methacrylate) (PHEMA) (Kim et al. 2018b), and polyvinyl alcohol (PVA) (Kanatt et al. 2012; Narasagoudr et al. 2020) has been adopted (Table 19.1). Among these films, CH-PVA are of great interest due to their remarkable mechanical properties (El-Hefian et al. 2010). However, the hydrophilicity of NH_2 groups from CH and OH groups in PVA decreased the water barrier characteristics of these films. Consequently, the incorporation of natural fillers or nanoparticles has been found suitable to develop packaging composite films with improved properties (Vadivel et al. 2019; Rubentheren et al. 2015). CH-based films/coatings with high potential applications in food packaging have been prepared by either solvent casting method (Bajić et al. 2019) or supercritical carbon dioxide and microwave techniques (Dutta et al. 2009). The novelty of the last ones consists in obtaining the films/coatings without inclusion of any cross-linker or plasticizer (Dutta et al. 2009).

The antioxidant potential of CH films is relatively lower than that of phenolic compounds (Taghizadeh and Bahadori 2014; Ferreira et al. 2014; Kanatt et al. 2012; Moradi et al. 2012). The mechanism of antioxidant activity of CH is still disputable (Schreiber et al. 2013) because some studies indicated in vitro antioxidant properties of CH itself (Park et al. 2004; Yen et al. 2008), while many others have clearly shown low or no antioxidant activity of native CH (Casettari et al. 2012; Xie et al. 2001; Xing et al. 2005). The antioxidant activity of CH was generally attributed to its metal ion chelation efficiency (Guibal 2004) which prevents initiation of lipid oxidation. In addition, CH films/coatings reduce the rate of oxidation of packaged food simply by preventing their contact with oxygen, acting as a secondary antioxidant (CH films have low oxygen permeability of 0.08–31.07 cm^3 m/mm^2 day atm) (Caner et al. 1998; Georgantelis et al. 2007). As it was mentioned above, the phenolic compounds are known as primary antioxidants capable of hydrogen atom or electron donating with the stabilization of the resulted phenoxyl radicals through delocalization of unpaired electrons around the aromatic ring (Schreiber et al. 2013). Therefore, to improve the antioxidant activity of CH, a plethora of active phenolic compounds (Table 19.1) (Mathew and Abraham 2008; Rivero et al. 2010; Rubentheren et al. 2015; Sun et al. 2014) identified in grape seed extracts (Sogut and Seydim 2018), propolis (Rollini et al. 2017), flavones (Bi et al. 2020), curcumin (Vadivel et al. 2019), thinned young apple polyphenols (Sun et al. 2018), plant extracts (Bajić et al. 2019), β-Carotene (Haria et al. 2018), black chokeberry pomace extracts (Halász and Csóka 2018), tannic acid (Jing et al. 2019), gallic acid (Sun et al. 2014), rutin (Narasagoudr et al. 2020), apple peel polyphenols (Riaz et al. 2018), and green tea extracts (Siripatrawan and Noipha 2012; Wang et al. 2013) have been incorporated within CH-based systems. The effect of the active compounds included in CH-based films/coatings on the properties of the final system is presented in Table 19.2.

Other studies indicated the use of phenolic compounds as cross-linkers (Mathew and Abraham 2008; Rivero et al. 2010) or plasticizers to improve the tensile strength or the brittleness issues of CH films (Sun et al. 2014). As Table 19.2 shows the incorporating of different types of antioxidant ingredients, the antioxidant and antimicrobial activities of the films or coatings have been considerably improved. For instance, the addition of D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) has enhanced the solubility of flavonoids (Zhao et al. 2019), and the miscibility of baicalein (Yong et al. 2020). Bi et al. (2020) further demonstrated the TPGS interaction with CH matrix through hydrogen bonds as well as the enhancement of the water vapor and gas barrier properties of CH films. In addition,

Table 19.2 Bioactive materials based on polysaccharides and their features

Composition of polysaccharide-based materials	Extract type	The effect of the active compounds encompassed in polysaccharide-based materials on the properties of the final material
CH-based films (Sogut and Seydim 2018)	Grape seed extract (GSE)	Higher WVP and opacity and lower transparency, lightness, and tensile strength; antioxidant activity and antimicrobial effect against *E. coli, L. monocytogenes, S. aureus, and P. aeruginosa*
CH and D-α-tocopheryl PEG 1000 succinate composite films (Bi et al. 2020)	Flavones	The CH-based films containing luteolin showed the highest water vapor, oxygen, and UV light barrier properties, tensile strength, and antioxidant activity. The CH-based films containing chrysin exhibited the lowest water vapor, oxygen, and UV light barrier properties and tensile strength but the highest antibacterial activity against *E. coli, S. typhimurium, S. aureus, and L. monocytogenes*
PVA/CH/nHA hybrid film (Vadivel et al. 2019)	Curcumin	Antioxidant activity and ammonia color sensing to monitor fish freshness. Antimicrobial and antifungal activities against *E. coli* and *S. aureus, A. niger,* and *A. flavus*
CH films (Sun et al. 2018)	Thinned young apple polyphenols (YAP)	Retard the increase in CFU, PV, TBARs, TVB-N, pH, and b* values that were caused by microbial reproduction and oxidation of lipids and proteins in GCF during cold storage
CH films (Bajić et al. 2019)	Natural plant extracts	Improved optical properties reflected in the ability to completely block the light transmittance at the wavelengths below ~330 nm; antibacterial activity against *Bacillus subtilis*
CH-gelatin films (Haria et al. 2018)	β-Carotene	The addition of β-carotene-loaded starch nanocrystals into CH-gelatin films resulted in lower values for swelling index, solubility in water, degradation, and moisture content when compared to those of control CH-gelatin films. The modified films showed 91.5 ± 0.3% of radical-scavenging activity

(continued)

Table 19.2 (continued)

Composition of polysaccharide-based materials	Extract type	The effect of the active compounds encompassed in polysaccharide-based materials on the properties of the final material
CH (Jing et al. 2019)	Tannic acid (TA)	TA-CH films showed a good solubility in water after conjugation as well a much stronger free radical-scavenging activity and ferrous ion chelating activity than CH films
CH/PVA (Kanatt et al. 2012)	Mint extract (ME)/ pomegranate peel extract (PE)	ME/PE extracts introduced excellent antioxidant activities and improved the tensile strength of the films without a significant effect on their barrier properties; antibacterial activity against gram-positive food pathogens
CH (Riaz et al. 2018)	Apple peel polyphenols (APP)	The thickness density, swelling degree, solubility, and opacity of CH film were enhanced, but the water content and vapor permeability were declined, suggesting that water barrier property of the films was considerably improved; antimicrobial effects on bacteria: *E. coli, B. cereus, S. aureus,* and *S. typhimurium*
CH (Siripatrawan and Noipha 2012)	Green tea extract	Maintained the physical and organoleptic qualities of the sausages by retarding the lipid oxidation and microbial growth better than CH-alone film. Antimicrobial and antioxidant properties of the CH films were enhanced by incorporation of the green tea extract
CH films (Sun et al. 2014)	Gallic acid	The incorporation of gallic acid significantly increased the antimicrobial activities of the films against *E. coli, S. typhimurium, L. innocua,* and *B. subtilis,* but the WVP and the oxygen permeability decreased
CH (Wang et al. 2013)	Tea polyphenols (TP)	WVP of the CH film was reduced, and the antioxidant activity of the CH film was greatly improved after addition of TP; and also a better ability to hinder the transmission of moisture and enhanced oxidation resistance was observed

(continued)

Table 19.2 (continued)

Composition of polysaccharide-based materials	Extract type	The effect of the active compounds encompassed in polysaccharide-based materials on the properties of the final material
CH/cellulose (Yu et al. 2013)	Caffeic acid	Superior antioxidant and antibacterial activity compared to the caffeic acid-free films
CH (Ferreira et al. 2014)	Grape pomace extracts	Enhanced antioxidant properties without modification of their water solubility and mechanical properties
CH (Rubilar et al. 2013)	Carvacrol and grape seed extract	Improved antimicrobial and antioxidant activity against *P. aeruginosa, S. aureus, L. innocua, E. faecalis, and S. cerevisiae*
ALG/PEO-blended nanofibers (Surendhiran et al. 2019)	Phlorotannin	Damaged the physical and morphological integrity of bacteria as well as their macromolecules (ATP, DNA, and proteins) leading thus to cell death. The mats composed of electrospun fibers with phlorotannin habe been proposed as excellent candidates to restrict the microbial food spoilage (antimicrobial activity against *S. enteritidis*)
Gelatin-NaALG-based films (Ho et al. 2020)	Pseuderanthemum palatiferum (Nees) Radlk	Improved antioxidant activities and light resistance of the films with low WVP
ALG films (Kim et al. 2018a)	Black chokeberry extract	Biodegradable active films with antioxidant abilities
ALG (Shankar et al. 2019)	Mixture of essential oils and citrus extract	Combination treatments of ozonation or gamma irradiation with ALG/EOs coating improved the shelf life of low-fat content white fillets of Merluccius sp. and were very effective in eliminating microflora
CaALG hydrogels (Biao et al. 2019)	Tea polyphenols	The tensile strength, breaking strain, and WVP of the films increased as the tea polyphenol level increased whereas the optical transmittance decreased. The antioxidant capacity of the films and the anti-inflammatory properties were enhanced
ALG/PEO composite nanofibers (Gutierrez-Gonzalez et al. 2020)	Curcumin (CU)	CU was successfully encapsulated in the SA nanofibers, and composites with excellent mechanical properties were obtained

(continued)

Table 19.2 (continued)

Composition of polysaccharide-based materials	Extract type	The effect of the active compounds encompassed in polysaccharide-based materials on the properties of the final material
NaALG (Olivo et al. 2020)	Turmeric	The use of 1% turmeric alcoholic solution as antimicrobial agent was not effective to reduce coliforms in cheeses with high contamination. The coated cheeses had higher microbial growth although the total coliform bacteria were reduced according to the storage time. Coverage significantly altered hardness, gumminess, chewiness, and cohesiveness over time, while elasticity was not affected
Polyurethane/DEX membranes (Sheet et al. 2018)	Pyocyanin	The bacterial inhibition tests revealed that the antibacterial activity of electrospun mat containing pyocyanin was 98.54 and 90.2% toward *E. coli* and *S. aureus*, respectively
PL films (Esposito et al. 2020)	Polyphenolic-rich extracts from chestnut spiny burs and roasted hazelnut skins	Antibacterial activity toward *S. aureus* demonstrated antibacterial effectiveness with the increasing content of both hydroalcoholic extracts. PL-based films exhibited a minimum antioxidant activity
Gelatin films (Ge et al. 2018)	Rosmarinic acid (RosA)	The RosA-gelatin edible films presented good water resistance property, strong mechanical property, excellent ultraviolet light barrier ability, and long-term antibacterial activity (*E. coli* and *S. aureus*)
XG coatings (Galindo-Pérez et al. 2015)	Tocopherol	Polymeric nanoparticles significantly reduced initial respiration rates with respect to controls and decreased the enzymatic catalytic activity
XG (Golly et al. 2019)	Ascorbic and citric acid	The ascorbic and citric acid enriched XG coatings preserved the color, phytochemicals, antioxidant, and textural properties of grapes in cold storage

(continued)

Table 19.2 (continued)

Composition of polysaccharide-based materials	Extract type	The effect of the active compounds encompassed in polysaccharide-based materials on the properties of the final material
XG microparticles (Outukia et al. 2016)	Extract of Eschweilera nana Miers leaves	The *E. nana* extracts and the microparticles loaded with *E. nana* extracts showed significant ability to inhibit the release of radical oxygen species (ROS) in the neutrophil respiratory burst
XG-based edible coating (Sharma and Ramana Rao 2015)	Cinnamic acid	The incorporation of cinnamic acid as antioxidant agent into XG-based edible coatings caused retardation of the oxidative browning, declining the ascorbic acid level, degradation of total phenolics content, and reduction in antioxidant capacity as compared to fresh-cut pears coated only with XG and uncoated ones
XG/lignin (Raschip et al. 2020a)	Vanillin	The XG-lignin films containing an aspen wood lignin type showed a high antibacterial activity against *S. typhimurium*, *E. coli,* and *L. monocytogenes* bacteria compared with those containing softwood lignin or lignin from annual plants. The vanillin release rate dependence on the lignin type was also evidenced, a retarded release of vanillin being observed
XG/PVA films (Raschip et al. 2020b, c)	Red grape pomace (RGP)	Improved antioxidant and antimicrobial activity against *S. typhimurium, E. coli,* and *L. monocytogenes* of the XG/PVA/RGP cryogel films compared to the XG/PVA films. The swelling studies revealed a reduced water permeability for RGP-enriched XG/PVA films

*b** yellow/blue values, *CH* chitosan, *CFU* colony-forming units, *EOs* mixture of essential oils and citrus extract, *GCF* grass carp fillets, *nHA* hydroxyapatite, *PEG* polyethylene glycol, *PEO* polyethylene oxide, *PV* peroxide value, *PVA* polyvinyl alcohol, *TBARs* thiobarbituric acid, *TVB-N* total volatile basic nitrogen by embedding of flavonoids into CH-TPGS films, the antioxidant and antimicrobial activities of the films were remarkably enhanced (Bi et al. 2020).

19.2.2 Alginate

Alginate (ALG) is an anionic polysaccharide (Table 19.1) obtained from seaweed (Khalil et al. 2017), being composed of (1,4)-β-D-mannuronic acid (M) and α-L-guluronic acid (G) units (Cazon et al. 2017). The physical properties of ALG are affected by the M/G ratio (Bekin et al. 2014). Thus, high proportion of G unit generates stable and ordered 3D networks with eggbox structure endowed with enhanced toughness (Sari-Chmayssem et al. 2016). The ALG-based gels with low amount of G unit exhibited an increased elasticity.

The ALGs prepared from seaweed are highly soluble in water; therefore, they exhibit poor water barrier properties. This disadvantage can be overcome by cross-linking with polyvalent cations, especially Ca^{2+} ions (Benavides et al. 2012; Gomaa et al. 2018). Combination of two biopolymers (Ho et al. 2020; Namratha et al. 2020) or blending with synthetic polymers (Surendhiran et al. 2019) has been also reported. Cross-linked ALG films are generally obtained by solvent casting method, and although the ALG-based films/coatings exhibit poor water barrier properties, their hygroscopicity delays the dehydration of food (Cazon et al. 2017). Furthermore, to confer antioxidant activity to ALG-based films, the entrapping of black chokeberry extracts (Kim et al. 2018a), *Pseuderanthemum palatiferum (Nees) Radlk* (Ho et al. 2020), or turmeric (Olivo et al. 2020) has been proposed (Table 19.2). Electrospun nanofibers consisting of NaALG-PEO encapsulating phlorotannin (Surendhiran et al. 2019) or curcumin (Gutierrez-Gonzalez et al. 2020) with enhanced antimicrobial and antioxidant properties were also recently described.

19.2.3 Xanthan

Xanthan gum (XG), a microbial polysaccharide, comprises of a β-(1–4)-D-glucose, in which every alternate glucose residues has a three-sugar side chain comprising of two mannose residues with a glucuronic acid residue between them (Table 19.1). The mannose residues are substituted at positions C4 and C6 by generally 30–40% pyruvate group (Raschip et al. 2020a). The fermentation conditions are mainly the factors affecting the percentage values of pyruvate and acetate groups. Toxicological tests have been performed to asses XG safety (Bejenariu et al. 2009; Dumitriu and Dumitriu 1991), and it was demonstrated that XG is nontoxic and does not cause inflammation or irritation of the skin or eyes. On this basis, XG has been approved by FDA for use as food additive (Petri 2015). In 1980, XG was included as E-415 in the food emulsifier list. Therefore, a large variety of XG-based materials in form of films, coatings, hydrogels, or cryogels have been synthesized with the aim of encapsulating and controlling nutrition ingredients and therapeutic agents release in food or biomedical applications (Huang et al. 2018; Kulkarni and Sa 2008; Li and Nie 2016; Petri 2015; Popa et al. 2010; Tao et al. 2016; Raschip et al. 2020a, b, c).

The physicochemical properties of some XG-based hydrogels have been improved by embedding various types of lignins (Raschip et al. 2007, 2011, 2013). We extend our studies, and we recently designed new XG/lignin films containing vanillin, one of the most widely used flavoring agent with antimycotic and bacteriostatic properties (Raschip et al. 2020a; Vasile et al. 2013). Vanillin is also known as an effective antioxidant in foods containing polyunsaturated fatty acids, and its great potential in keeping quality of dried foods has been proven (Burri et al. 1989; Tai et al. 2011). The vanillin effectiveness in suppression of *E. coli* and *L. monocytogenes* bacteria growth in “Granny Smith” apple juice, yeasts, and molds has been also demonstrated (Moon et al. 2006). In own studies, XG hydrogel films containing various types of lignin were confirmed to display remarkable antibacterial activity against *S. typhimurium, E. coli,* and *L. monocytogenes* (Raschip et al. 2020a) bacterial spoilage organisms. To improve the water barrier properties of XG/PVA-based films, the cryogelation methodology through freeze/thawing cycles (Lozinsky 2002) has been adopted to design physically cross-linked composite films (Zhang et al. 2019; Raschip et al. 2020b, c). These materials present highly interconnected macroporous morphologies formed as a result of freezing (Lozinsky 2018). By embedding red grape pomace (RGP) extracts within the XG/PVA-based cryogel matrices (Fig. 19.1a), novel bioactive packaging materials endowed with antioxidant features have been obtained (Raschip et al. 2020b, c).

The RGP entrapped within XG/PVA-based cryogel matrices (Fig. 19.1b) was visually proved by changing the color of the films. Thus, the colors of the XG/PVA-based cryogel matrices turned from white (**CG1**, Fig. 19.1b) to dark pink (**CG3**, Fig. 19.1b), for the sample with the highest RGP content. The ratio between polymeric components, the number of freeze/thawing cycles, and the freezing/thawing temperatures have been found to be the key parameters affecting the morphology and swelling properties of the bioactive cryogels (Raschip et al. 2020b). The SEM micrographs (Fig. 19.1c) indicated a heterogeneous porous structure with interconnected pores for XG/PVA cryogels. The corresponding pore distribution diagrams (Raschip et al. 2020b) showed a decrease of the average pore sizes with the increase of the amount of antioxidant incorporated within XG/PVA network (sample CG3, inset of Fig. 19.1c).

Recently, Golly et al. (2019) focused on shelf life extension of Pinot noir grapes under cold temperature storage using XG-based coatings enriched with ascorbic (AS) and citric (CI) acids (Table 19.2). It was showed that the CI-enriched XG-based coatings were the best in preserving the texture, color, and antioxidant properties of Pinot noir grapes under cold temperature storage. In the research done by Sharma and Ramana Rao (2015), XG-based coatings enriched with cinnamic acid prevented browning and extended the shelf life up to 4 days and 8 days of fresh-cut “Nashpati” and “Babugosha” pears, by inhibiting the enzymes activity and thus delaying the browning incidence. XG microparticles loaded with Eschweilera nana Miers leaves extract obtained by spray-drying technique developed a remarkable improved antioxidant capacity (Outukia et al. 2016).

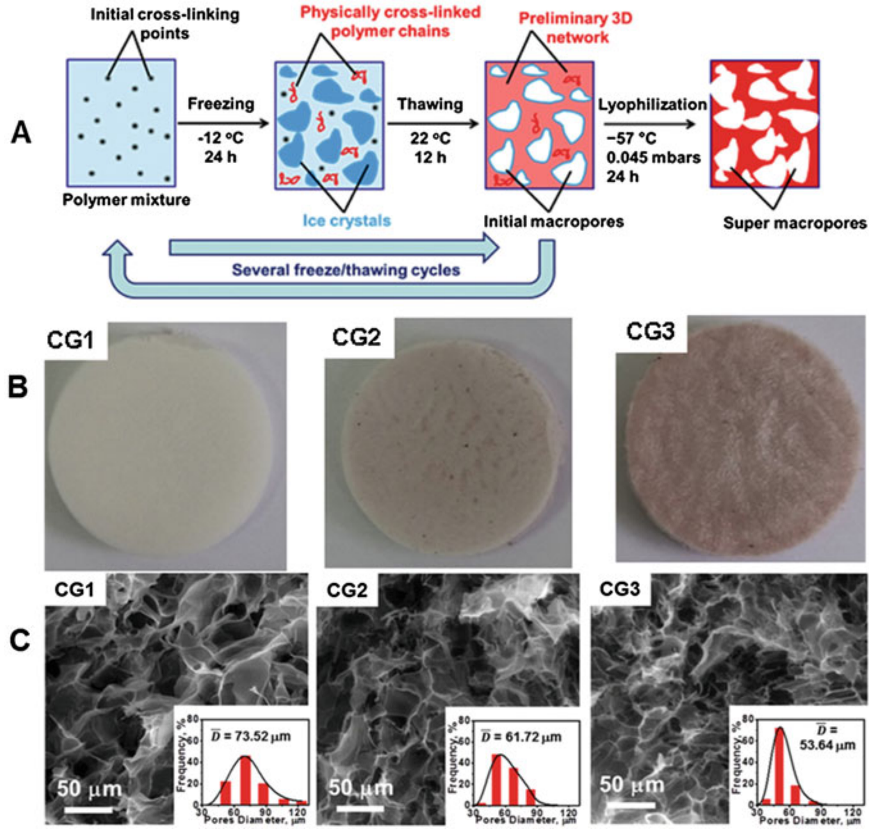


Fig. 19.1 (**a**) Schematic representation of the cryogenic gelation of the polymer components mixture in water through freeze/thawing cycles. (**b**) Optical images of the XG/PVA cryogels prepared without RGP (CG1) and with an RGP content of 0.2 w/w% (CG2) and, respectively, of 0.5 w/w% (CG3). (**c**) SEM micrographs and the corresponding pores distribution diagrams for XG/PVA cryogels. (Reproduced from Raschip et al. 2020b, Copyright 2020, with permission from Elsevier)

19.2.4 Dextran

Dextran (DEX) is a hydrophilic, biocompatible, and biodegradable polymer composed of linear-l,6-linked D-glucopyranose residues (Table 19.1) (Dinu et al. 2011), being obtained by the action of dextransucrase, on sucrose (Kothari et al. 2014). The commercially available DEX is synthesized from *Leuconostoc mesenteroides* (Robyt et al. 2008; Siddiqui et al. 2014). The source of strains and the cultivation conditions are the major factors affecting the molar mass, the spatial arrangement, the type, and the degree of branching of DEXs (Wang et al. 2020). DEXs linear with a high molecular weight are widely utilized in bakery formulations (Lacaze et al. 2007). DEXs are capable to enhance dough rheological properties, including dough strength and water sorption capacity due to their high capability to hold high amounts of water (Wang et al. 2018, 2019; Zannini et al. 2014). DEXs have been also granted as inhibitors reducing the bread palatability during storage and thus attending a longer shelf life (Wang et al. 2019; Zhang et al. 2018).

The development of novel antibacterial polyurethane-dextran (PU-DEX) electrospun nanofibers by entrapping pyocyanin extracted from *P. aeruginosa* has been recently pointed out (Sheet et al. 2018). The composite nanofibrous membranes with unique solubility characteristics and mechanical and swelling properties were obtained by electrospinning after mixing solution in organic solvents. The addition of pyocyanin was demonstrated to be beneficial, and the hybrid electrospun mat showed an antibacterial activity of 98.54 and 90.2% against *E. coli* and *S. aureus*, respectively (Sheet et al. 2018). In another study, a curcumin-dextran conjugate (CUM-DEX) has been prepared by free radical grafting reaction between CUM and DEX, and its remarkable antioxidant and antimicrobial properties against food pathogens has been demonstrated (Zare et al. 2019). In addition, the experiments for evaluation of antitumoral characteristics of CUM-DEX conjugate indicated a reliable potential of these materials in further biomedical applications (Zare et al. 2019).

19.2.5 Pullulan

Pullulan (PL), an extracellular microbial polysaccharide originated from the fungus-like yeast, *Aureobasidium pullulan* (Wu 2016), comprises of maltotriose units interconnected to each other by an α-(1,6) glycosidic bond (Table 19.1) (Chen et al. 2015). PL has several unique features including high adhesion, adherence, and film-forming abilities and reduce wear and friction (Kong and Ziegler 2014; Kraśniewska et al. 2019), which recommend its usage in food coatings, as flocculant, lubricant, foaming agent, or adhesive (Aceituno-Medina et al. 2013; Tabasum et al. 2018). PL could be also used as carrier for bioactive compounds or as safeguarding packaging for food and pharmaceutical products. However, the PL drawbacks such as brittleness, high cost (Tong et al. 2008; Lin et al. 2007), low mechanical strength (Kristo and Biliaderis 2007), and the lack of antimicrobial properties (Campos et al. 2011) limit its use at large scale. Consequently, PL is combined with different natural or synthetic polymers, including carrageenan, CH, ALG, starch, hyaluronic acid, carboxymethyl cellulose (CMC), polyethyleneimine (PEI), polyisopropylacrylamide (PNIPAm), polyamidoamine, and PVA. The applications of PL-based blends have been recently reviewed (Tabasum et al. 2018). For instance, an extended shelf life of fresh-cut pineapples was achieved using edible coatings which contain PL, CH, and various natural compounds extracted from cactus and aloe (Treviño-Garza et al. 2017). Wu and Chen (2013) demonstrated that edible films based on the PL and mixtures of chitooligosaccharide and glutathione have been indicated to prolong the shelf life of apple slices. PL-based films containing nano-SiO_2 and whey protein isolate were also shown to enhance the shelf life of meals and were proposed as eco-friendly promising alternatives to plastic-based packaging films (Hassannia-Kolaee et al. 2016). The formation of hydrogen bonds between OH groups of PL and the COOH groups of CMC had synergistically enhanced on the physicochemical properties of films (Shao et al. 2018), while the incorporation of tea polyphenols (TP) has been indicated as a very efficient way to preserve the quality of strawberries during storage (Shao et al. 2018).

The influence of tea polyphenols addition on physicochemical characteristics of curdlan/CH films (Zhou et al. 2019) or salecan/N,N,N-trimethyl chitosan PEC hydrogels (Hu et al. 2020) as well as on their applications to food preservation has been also recently described. Performance analysis showed that the obtained curdlan/CH composite films had a high tensile strength (Zhou et al. 2019). In addition, the curdlan/CH composite films reduced the gas exchange and the water loss inside and outside of the packaging. Adding tea polyphenols to curdlan/CH composite films significantly improved the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity, while the water vapor transmission (WVT) coefficient of films drastically decreased. Moreover, microorganism growth was inhibited when curdlan/CH/tea polyphenols composite films were tested as coatings during cold fresh meat storage. On the other hand, the degradation tests demonstrated that the PEC hydrogels are disintegrated in the presence of lysozyme (Zhou et al. 2019), while the cumulative release study of TP from PEC hydrogels indicated their huge potential for sustained release of nutrients.

19.3 Properties of Bio-Based Materials Embedding Natural Antioxidant Agents

The water vapors and gas transmission rates are two major factors that are necessary to be controlled in order to accomplish the appropriate quality and prolonged shelf life for humidity-sensitive foods. Therefore, films endowed with reduced moisture adsorption tendency, enhanced flexibility, optical transparency, gas barrier properties, thermal stability, mechanical toughness, biodegradability, and antioxidant and antimicrobial properties are highly demanded for food packaging applications (Cazon et al. 2017). Several research studies indicated the preparation of bioactive edible films based on polysaccharides combined with other natural and/or synthetic polymers or natural antioxidant agents (Table 19.1) endowed with advanced features (Table 19.2) (Wang et al. 2013; Bi et al. 2020; Biao et al. 2019). The moisture adsorption behavior of rutin (Rt)-loaded CH/PVA bioactive films (CPR) (Fig. 19.2a) was compared with that of control CH/PVA films (Narasagoudr et al. 2020).

The inclusion of Rt within CH/PVA films induced a significantly decrease in the humidity adsorption tendency, which was assigned to the formation of hydrogen bonds between the functional of the film components. Furthermore, the transparency results (Fig. 19.2b) clearly revealed that Rt-loaded CH/PVA films blocked both UV and visible light (Narasagoudr et al. 2020), supporting their excellent UV light barrier properties.

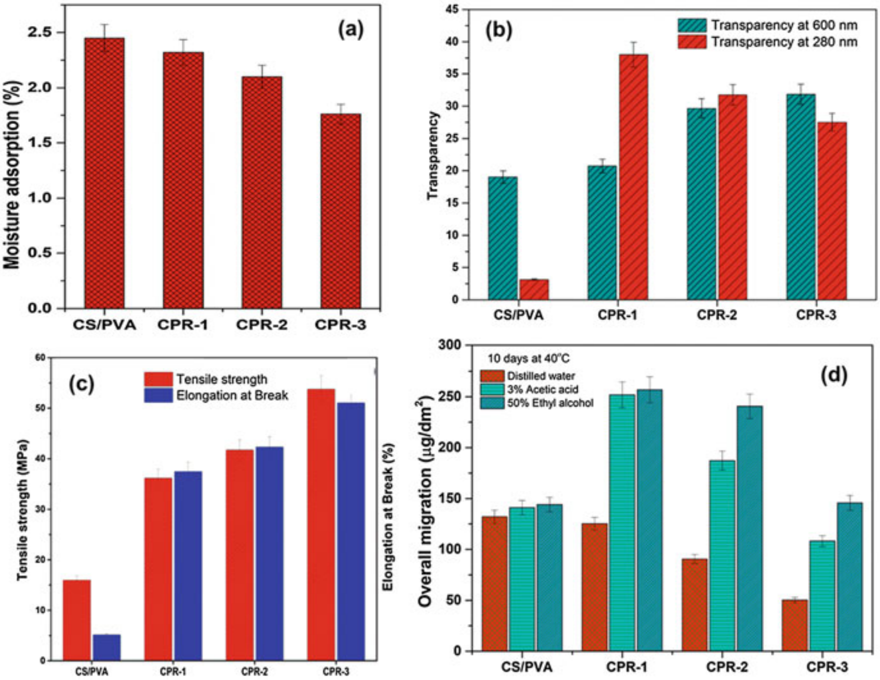


Fig. 19.2 Hydrodynamic, optical, and mechanical properties and toxicity evaluation through overall migration tests of control CH/PVA and CPR bioactive films: (**a**) moisture adsorption, (**b**) transparency, (**c**) tensile strength, and (**d**) migration of components of bioactive films in three different food simulants. (Reproduced from Narasagoudr et al. (2020), Copyright 2020, with permission from Elsevier)

19.3.1 Water Barrier Properties

The food packaging material must act as a barrier, stopping or diminishing to normal limits the penetration of light, temperature, or other physical agents resulting in a continuous deterioration of the quality of the products. The barrier role also aims the transfer of gas from the internal or external environment in order to avoid loss of product-specific flavorings and dehydration. If the packaging material does not provide an adequate barrier, microorganisms can contaminate food and make it unsafe. The main permeates investigated in food packaging applications are water moisture and oxygen (Siracusa et al. 2008; Cazon et al. 2017). The WVP represents the amount of water that pass through films per unit of area and time (kg/m s Pa) (Cazon et al. 2017) and is commonly used to evaluate the water vapor barrier properties of coatings. The pressure and thickness of the films are also considered when WVP is determined. The water vapor barrier features are important to be studied because dehydration of fresh vegetables could be prevented or the moisture uptake from the environment could be avoided in some other dry foods like bread (Cazon et al. 2017; Miteluț et al. 2015).

The WVP values of CH-based films are significantly influenced by the DD values of CH, pH of aqueous medium, and the acid nature used to prepare the CH solution (Kim et al. 2006). For example, the CH-based films prepared using CH with low DD showed the lowest values of WVP (Kim et al. 2006). Regarding the pH effect, the WVP values increased with pH value from 3 to 4. The WVP values of CH-based films prepared in formic or lactic acid were significantly higher than those obtained using acetic or propionic acid. The inclusion of starch into CH matrix diminished the values of WVP (Santacruz et al. 2015). The molar mass of CH did not affect the WVP values but had great influence on their mechanical properties (Kerch and Korkhov 2010).

The water vapor barrier properties are also changed by the incorporation of various antioxidant agents (Sun et al. 2014; Wang et al. 2013; Riaz et al. 2018). Thus, the entrapping of lower concentrations of gallic acid upgraded the barrier properties of CH film by lowering WVP and oxygen permeability (OP) by 11.1% and 58.5%, respectively (Sun et al. 2014). In another study, with the addition of apple peel polyphenols (APP), the swelling degree, solubility, and opacity of CH films were significantly raised, but the water content and WVP were diminished, indicating the enhancement of the water barrier property of the films (Riaz et al. 2018). The barrier properties and antioxidant activity of the CH-based polymeric matrix have been also improved by addition of starch nanocrystals (BS-NCs) and β-carotene during the CH film preparation (Haria et al. 2018). It was demonstrated that the addition of BS-NCs is beneficial especially for safeguarding of food from degradation by enzymes, acids, and salts.

The M/G ratio within ALG-based films has been found to influence the WVP properties (Olivas and Barbosa-Canovas 2008). Thus, the ALG-based films with a high content of G units proved to have better humidity barriers than those with a lower proportion of G units. A drastic decrease in WVP values of the ALG-based films was observed when a high concentration of Ca^{2+} ions was involved in the film gelation process (Benavides et al. 2012).

19.3.2 Mechanical Properties

The tensile strength (TS), the percentage of elongation at break (% EB), and the Young's modulus are the mechanical parameters commonly evaluated. The values of these parameters are generally determined as a function of the ratio between components and cross-linker degree or based on the synthesis medium conditions (Vieira et al. 2011). The evaluation of the mechanical properties is absolutely necessary because it anticipates the films' behavior under various food processing conditions (Fakhreddin Hosseini et al. 2013). The TS and EB values of Rt-loaded CH/PVA films were compared with those obtained for control CH/PVA films (Fig. 19.2c). The inclusion of 0.2–0.6% of Rt within CH/PVA films significantly improved the stiffness and flexibility of the bioactive films (Narasagoudr et al. 2020). This behavior was associated to the formation of strong intermolecular interactions between functional groups of the components through hydrogen bonds. The high mechanical stability of these films was further supported by the positive results obtained for the overall migration of components from Rt/CH/PVA bioactive films (Fig. 19.2d). This parameter was evaluated using three food simulants including distilled water, acetic acid, and ethanol with a concentration of 3% and 50%, respectively (Narasagoudr et al. 2020). The overall migration values of Rt/CH/PVA films were in the range of the overall migration limit (10 mg/dm^2), which indicates their great potential as nontoxic packaging materials.

As it was shown above for WVP, the DD, the nature of solvent pH of the aqueous medium, and the CH molar mass are also affecting the mechanical properties of CH-based films. CH-based films prepared in formic or lactic acid exhibited lower TS values than the films obtained in acetic or propionic acid (Kim et al. 2006; Park et al. 2002). The TS values drastically decreased when the pH values rose. The CH-based films prepared with CH having high DD were very sensitive to pH changes in comparison to those prepared with CH with low DD (Kim et al. 2006). An increase in molar mass of CH generated films with high values of TS and EB. The TS and EB values diminished when the citric acid was used as solvent (Park et al. 2002; Zivanovic et al. 2005). The temperature and the relative humidity have also exerted huge impact on TS and EB of CH-based films, while storage period exhibited modest influence (Kerch and Korkhov 2010). Blending of CH with natural or synthetic polymers, including xylan (Kamdem et al. 2019), starch (Kowalczyk et al. 2015), PVA (Kanatt et al. 2012), and polyethylene terephthalate (Torres-Huerta et al. 2014), has been reported to improve mechanical properties of CH films. For instance, adding xylan significantly increased the EB of the composite films, and samples with 20 to 25 wt.% xylan exhibited the highest TS and Young modulus values (Kamdem et al. 2019). On the other hand, the entrapping of essential oils into the CH-based formulations resulted in a significant decrease of the TS and elastic modulus values (Gutierrez-Pacheco et al. 2016).

The relative humidity is the main factor affecting the mechanical properties of ALG-based films (Rhim 2004). The TS values of ALG-based films decreased at a high relative humidity level, while the EB values increased in these conditions (Olivas and Barbosa-Canovas 2008). Therefore, it can be concluded that the water is acting as a plasticizer into hydrophilic films. Electrospun nanofibers made of ALG-polyethylene oxide (PEO) and curcumin (CUM) were successfully obtained using trifluoroacetic acid (TFA) as cross-linking agent (Gutierrez-Gonzalez et al. 2020). The mechanical properties, i.e., TS and EB, were improved by CUM addition and TFA cross-linking (Gutierrez-Gonzalez et al. 2020). The combination of two biopolymers has been also pointed out as a nice approach to enhance the ALG-based films features. In this case, the films have been synthesized using gelatin and NaALG to improve their weaknesses, brittleness, and TS properties (Dou et al. 2018; Ho et al. 2020). In addition, by incorporation of *Pseuderanthemum palatiferum (Nees) Radlk* freeze-dried powder within ALG, the final films have been endowed with antioxidant activity (Ho et al. 2020). The addition of $CaCl_2$ to ALG generated ALG-based films with a TS value of about 32 MPa and an EB of 10.20% (Kim et al. 2018a). However, the embedding of black chokeberry extract within ALG/$CaCl_2$ films lowered the TS values (Kim et al. 2018a).

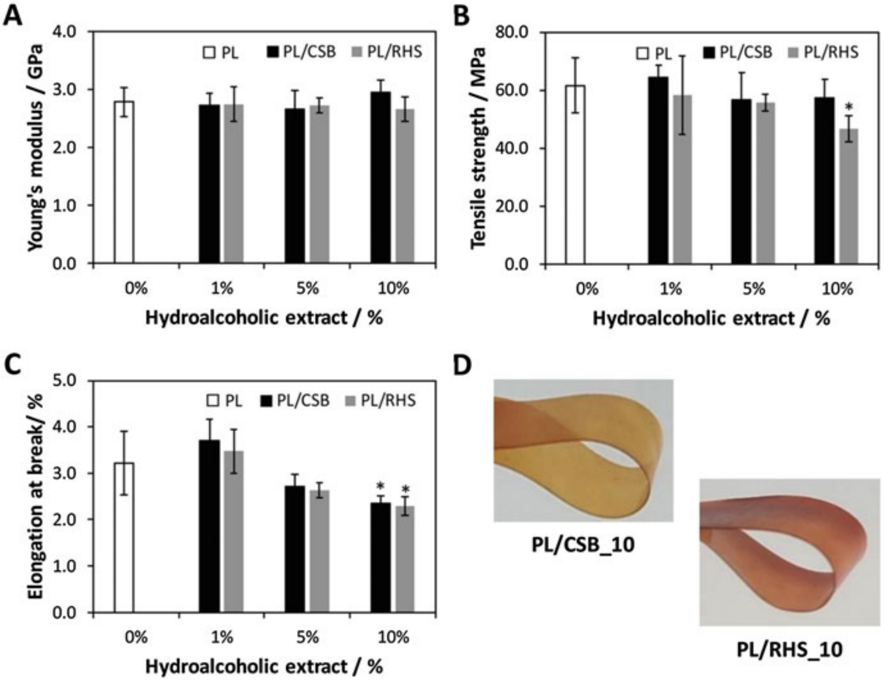


Fig. 19.3 (**a**) Young's modulus, (**b**) tensile strength, and (**c**) elongation at break of the PL, PL/CSB-, and PL/RHS-based films and (**d**) photographs demonstrating the flexibility of the films. All values are the mean of five replicates, and the error bars represent the respective standard deviations. The asterisk (*) denotes statistically significant differences with respect to the plasticized PL film ($p < 0.05$). (Reproduced from Esposito et al. (2020), Copyright 2020, with permission from Elsevier)

The mechanical performance of bioactive PL-based films loaded with polyphenolic-rich extracts from chestnut spiny burs (CSB) and roasted hazelnut skins (RHS) was also investigated by tensile tests in comparison to control PL films (Esposito et al. 2020). The plasticized PL film exhibited a Young's modulus of 2.79 GPa, TS of 61.6 MPa, and EB of 3.22% (Fig. 19.3). The embedding of the polyphenolic-rich extracts from CSB and RHS within the PL films had a moderate influence on the Young's modulus (Fig. 19.3a) and TS (Fig. 19.3b) values due to the low quantity of both extracts loaded into PL films. Regarding the EB (Fig. 19.3c), the addition of 10% of CSB or RHS extracts reduced this parameter to values of 2.35% for PL/CSB_10 and 2.29% for PL/RHS_10. Despite the low values of EB of both types of PL-based films, these materials were still adequately pliable and flexible as illustrated in the optical photographs of Fig. 19.3d (Esposito et al. 2020).

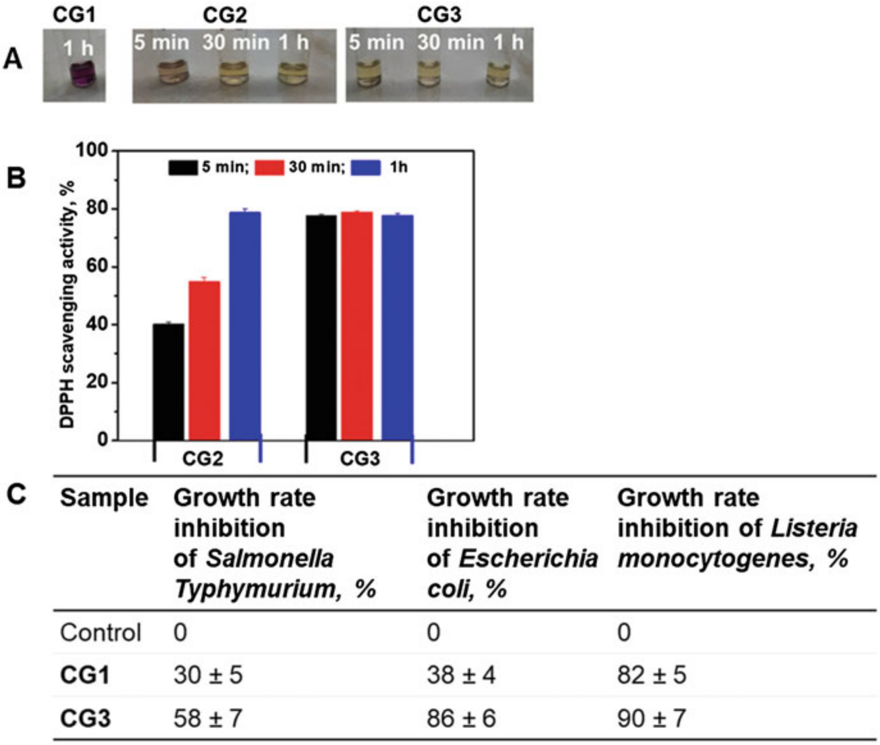
19.3.3 Antioxidant and Antimicrobial Properties

Smart packaging with antimicrobial support has been demonstrated to be an effectively safeguard against microbial growth (Raschip et al. 2020a; Campos et al. 2011; Misra et al. 2018).

In order to develop adequate antimicrobial edible materials, a series of characteristics such as interactions between food components and biopolymers, the physicochemical properties of films, must be taken into account to ensure protection of the quality of products (Campos et al. 2011). The antioxidant properties of the polysaccharide-based films are typically assessed by DPPH and ABTS radical-scavenging assays (Raschip et al. 2020b; Kim et al. 2018b). In the DPPH assay, the H-transfer reactions are monitored by UV-vis spectroscopy whereby the decay of the DPPH visible band at 517 nm is recorded. The DPPH solution changes its color from purple to pale yellow as a response to scavenging of the free radicals (Raschip et al. 2020b). The ABTS assay is based on the generation of a blue/green ABTS• + species that can be reduced by antioxidants (Kim et al. 2018b).

The CH-based films conjugated with gallic acid (Pasanphan and Chirachanchai 2008), catechin (Curcio et al. 2009), caffeic acid (Božič et al. 2013), and tannic acid (Božič et al. 2012; Jing et al. 2019) obtained by either carbodiimide-based coupling reaction or free radical grafting procedure or laccase-mediated reaction have been shown to exhibit enhanced antioxidant capacity. The gallic acid-functionalized CH-based hydrogels were capable to scavenge DPPH and ABTS free radicals since the hydrogen-donating capacity of the third hydroxyl group of gallic acid can stabilize the phenoxy radical (Kim et al. 2018b). Furthermore, higher antioxidant effects were observed on the gallic acid-functionalized hydrogels with the longer succinyl-CH. Similar improvement in the antioxidant activity was also obtained when trans-resveratrol, the natural compound identified in red grape pomace (RGP) extracts, was embedded into XG/PVA-based films (Raschip et al. 2020b). The control films (**CG1**) did not exhibit antioxidant activity (Fig. 19.4a, no color change detection).

The RGP-loaded XG/PVA cryogel films exhibited a DPPH scavenging activity with the increase of the RGP content (Fig. 19.4b). A DPPH scavenger activity higher than 77% was determined only after 5 min for **CG3** sample (0.5 w/w% of RGP). The formation of stable semiquinone radicals with electron delocalization over the entire molecule supported the strong scavenger potential of trans-resveratrol (Raschip et al. 2020b). The XG/PVA cryogel matrix embedding RGP (**CG3**) successfully hindered the growth of *Salmonella typhimurium*, *E. coli,* and *L. monocytogenes* bacteria (Fig. 19.4c) (Raschip et al. 2020b). The antibacterial activity of trans-resveratrol identified in the RGP extract could be associated with its iron chelating properties or proteins binding ability through hydrogen bonds (Bi et al. 2019; House 1999; Field and Lettinga 1992). The antibacterial effectiveness of CH-coated polypropylene (PPE) films incorporating propolis extracts (EEP) against *Bacillus cereus, Cronobacter sakazakii, E. coli, L. monocytogenes, Salmonella typhimurium, and S. aureus* foodborne pathogens has been also described (Torlak and Sert 2013). The results obtained showed that antimicrobial activity against both gram-positive and



Sample	Growth rate inhibition of *Salmonella Typhymurium*, %	Growth rate inhibition of *Escherichia coli*, %	Growth rate inhibition of *Listeria monocytogenes*, %
Control	0	0	0
CG1	30 ± 5	38 ± 4	82 ± 5
CG3	58 ± 7	86 ± 6	90 ± 7

Fig. 19.4 (**a**) Optical images of **CG1, CG2**, and **CG3** samples after performing the DPPH test. (**b**) DPPH scavenging activity of **CG2** and **CG3** samples evaluated after 5 min, 30 min, or 1 h of incubation in dark. (**c**) The growth rate inhibition of bacteria by XG/PVA cryogels without (**CG1**) and with RGP (**CG3**). (Reproduced from Raschip et al. 2020b, Copyright 2020, with permission from Elsevier)

gram-negative bacteria was enhanced with addition of EEP to CH-PPE film formulations. Hybrid composite films consisting of CH, xylan, PVA, hydroxyapatite (nHA), and curcumin, as antioxidant agent, with improved antimicrobial and anti-fungal against *A. Niger* and *A. Flavus* have been reported by Vadivel et al. (2019). These hybrid films presented comparable features with those reported for standard streptomycin and amphotericin (Vadivel et al. 2019).

A versatile method was developed to generate bioactive food packaging materials, which involves the inclusion of tea polyphenols (TP) into ALG-based films (Biao et al. 2019). The ALG and ALG/TP composite films were pliable and flexible with a homogeneous yellow-brown appearance (Fig. 19.5a), and the color intensity of the films increased with the increase of the level of TP added. Compared to the control, the ALG-based films containing 5.0% TP had about a 7.1-fold and, respectively, 41.0-fold higher DPPH and ABTS radical scavenge activity. Moreover, the addition of TP to the ALG films caused a drastic decrease in nitric oxide (NO) production in the cells (Fig. 19.5b). NO is typically used as

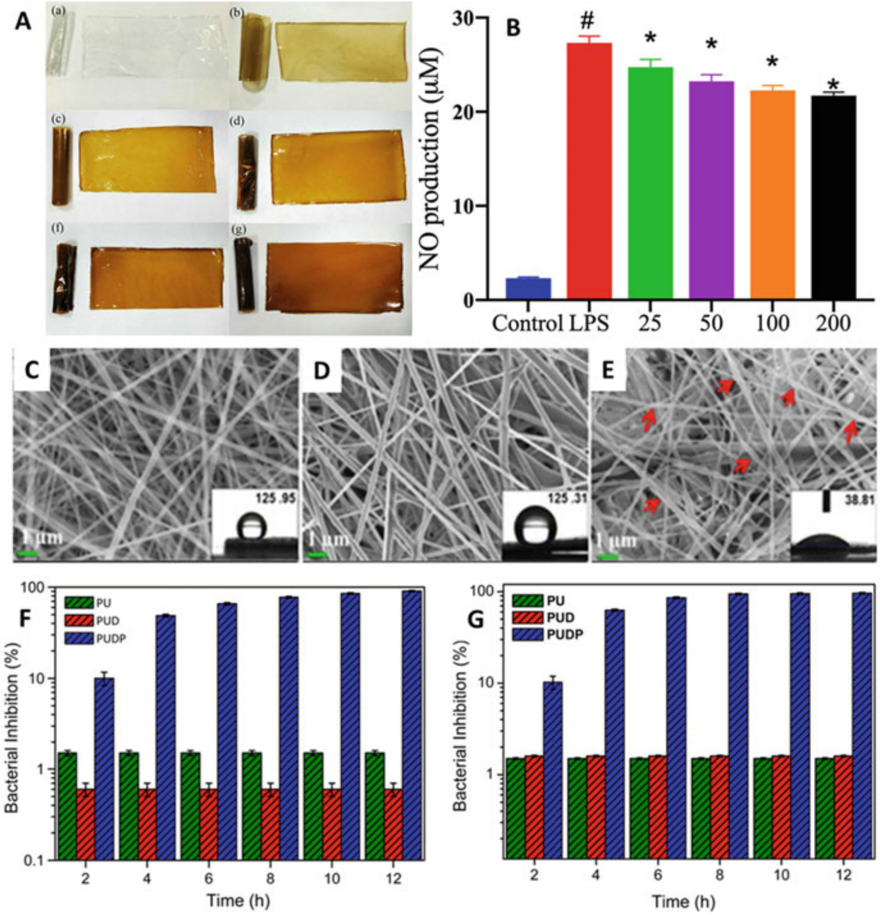


Fig. 19.5 (**a**) Photographs of ALG-based films with different tea polyphenol levels. *Key*: a, b, c, d, and e correspond to a content of tea polyphenol of 1%, 2%, 3%, 4%, and 5%, respectively. (**b**) Effect of ALG-based films with different tea polyphenol levels on LPS-stimulated NO production in RAW264.7 macrophages. The asterisk * indicated statistically significant difference of $p < 0.05$. # represents significantly difference from control group (reproduced from Biao et al. (2019), Copyright 2019, with permission Elsevier). FE-SEM images of mats of (**c**) polyurethane (PU), (**d**) polyurethane/dextran (PUD), and (**e**) polyurethane/dextran/pyocyanin (PUDP). Insets of (**c**–**e**) corresponding water contact angle images. Red arrows refer to irregular twisted and wrinkled bend after incorporating pyocyanin. (**f**) Growth inhibition of *E. coli* and (**g**) growth inhibition of *S. aureus* on PU and hybrid mats. (Reproduced from Sheet et al. (2018), Copyright 2018, American Chemical Society, Open Access Paper)

indicator of inflammation. For example, embedding of 25, 50, 100, or 200μg/mL samples decreased NO overproduction by 8.2%, 9.4%, 17.1%, and 25.7%, respectively, indicating strong anti-inflammatory properties for ALG/TP films besides their remarkable antioxidant activities (Biao et al. 2019).

Phlorotannin encapsulated in NaALG-PEO nanofibers was found to exhibit an excellent antimicrobial activity against *S. enteritidis*, one of the biggest challenging pathogens to the poultry industry (Surendhiran et al. 2019). The mechanism of action of phlorotannin on *S. enteritidis* bacteria was systematically analyzed and demonstrated that the phlorotannin was capable to destroy the physical and morphological integrity of bacteria as well as the ATP and DNA. Furthermore, phlorotannin-loaded NaALG-PEO nanofibers showed excellent antioxidant activity which contributes to the preservation of sensory quality of chicken at an adequate level, indicating the huge potential of these materials for food packaging applications (Surendhiran et al. 2019).

Hybrid nanofibrous mats consisting of polyurethane (PU), DEX, and pyocyanin were fabricated using electrospinning method, and their properties were compared with those of pristine materials (Sheet et al. 2018). The SEM micrographs showed flat ribbonlike structure, ellipse shape structure, and round fibers with a twisted and wrinkled bend morphology for PU nanofibers (Fig. 19.5c), PU/DEX nanofibers (Fig. 19.5d), and pyocyanin-loaded mats (Fig. 19.5e), respectively. From the insets of Fig. 19.5c–e, it can be observed that hydrophilic character of PU was enhanced after blending with DEX and pyocyanin, which facilitates the attachment of bacterial cells during antibacterial applications. The percentages of bacterial inhibition for the PU/DEX/pyocyanin mats were found to be about 98.54% for *E. coli* (Fig. 19.5f) and 90.2% for *S. aureus* (Fig. 19.5g), being significantly higher than those obtained for PU and PU/DEX nanofibers. This behavior was correlated with pyocyanin which is able to interrupt the active metabolic transport process of bacteria by its interaction with the cell membrane (Baron and Rowe 1981) or by oxidative stress (Jayaseelan et al. 2014).

19.4 Applications of Bio-Based Materials Embedding Natural Antioxidant Agents

Polysaccharide-based materials, as coatings or films, embedding natural antioxidant agents have been mainly applied to prolong the shelf life of foodstuff and to maintain their quality. Table 19.3 presents a series of studies performed using CH, ALG, PL, or XG-based coatings, films, hydrogels, or nanofibers in food products applications. The effects of CH and grape seed extract (GSE)-based edible films on the quality of vacuum-packaged chicken breast fillets have been investigated by Sogut and Seydim (2018). These films were proved to restrict the microbial development and lipid oxidation under refrigerated conditions (Sogut and Seydim 2018). CH films containing GSE and carvacrol increased the shelf life of refrigerated salmon by 4–7 days of storage (Alves et al. 2018). Other works demonstrated that GSE-incorporated CH films efficiently hindered the growth of mesophilic aerobic and coliform bacteria in fresh chicken breast fillets (Moradi et al. 2012; Rubilar et al. 2013), thus extending their shelf life. A possible system for fish freshness monitoring acting as a natural pH indicator has been obtained from CH, PVA, xylan, nHA, and CUM (Vadivel et al. 2019). The hybrid composite films were used in this study to

Table 19.3 Bioactive polysaccharide-based films and their reported effects on food products

Composition of the bioactive polysaccharide-based films	Food product	Reported effects on shelf life and food preservation
GSE incorporated into CH (Sogut and Seydim 2018)	Chicken breast fillets	Efficiently restricted the growth of mesophilic aerobic and coliform bacteria in fresh chicken breast fillets, and CH reduced the oxidation values in chicken breast fillets during refrigerated storage, thus extending their shelf life
Curcumin incorporated into PVA/CH/nHA hybrid film (Vadivel et al. 2019)	Indian oil sardine (*Sardinella longiceps*) fish	Sensitive detection of fish freshness
YAP incorporated into CH films (Sun et al. 2018)	Grass carp (*Ctenopharyngodon idellus*) fillets	Cold preservation of freshwater fish fillets
CH incorporating green tea extract (Siripatrawan and Noipha 2012)	Pork sausages	Maintain qualities and extend shelf life of the refrigerated pork sausages
CH/cyclodextrin incorporating carvacrol (Higueras et al. 2014)	Fresh chicken	Antimicrobial effect depending on the size of the film and storage time. Increased concentration of carvacrol affected sensorial attributes of chicken meat
Phlorotannin encapsulated in ALG/PEO-blended nanofibers (Surendhiran et al. 2019)	Chicken meat	Phlorotannin-encapsulated nanofibers were able to maintain the sensory quality of the chicken by effectively inhibiting the pathogens (*S. enteritidis*)
ALG containing a mixture of essential oils and citrus extract (Shankar et al. 2019)	Merluccius sp. fillets	The desirable shelf life of the fish fillets was achieved by modulating the EOs concentration as well as the doses of ozonation and gamma irradiation
NaALG with turmeric coating (Olivo et al. 2020)	Ripened cheeses	The tested solution did not effectively improve microbiological quality; however, coated cheese samples showed increased lactic acid bacteria, water activity, and improved cheese texture, making them softer, with less elasticity, cohesion, and chewing
Polyphenols-loaded PL-CMC electrospun nanofibers (Shao et al. 2018)	Strawberries	The PL-CMC-TP nanofibers significantly decreased weight loss and maintained the firmness of the strawberries and improved the quality of the fruit during storage
Tocopherol nanocapsules/XG coatings (Galindo-Pérez et al. 2015)	Apples	Polymeric nanoparticles containing α-tocopherol and incorporated into a polymeric matrix (XG) had a great potential for preserving the

(continued)

Table 19.3 (continued)

Composition of the bioactive polysaccharide-based films	Food product	Reported effects on shelf life and food preservation
		physicochemical characteristics of fresh-cut red delicious apples during 21 days of storage
XG-enriched with ascorbic and citric acid (Golly et al. 2019)	Grapes (pinot noir)	Acid enriched XG coatings preserved phytochemicals, color, antioxidant, and texture properties of grapes in cold storage
XG-based edible coatings enriched with cinnamic acid (Sharma and Ramana Rao 2015)	Pears	XG-based edible coatings containing cinnamic acid contributed to diminish the surface browning and to enhance the shelf life of fresh-cut "Nashpati" and "Babugosha" for 4 days and 8 days, respectively, at 4 °C

EOs mixture of essential oils and citrus extract, *YAP* young apple polyphenols

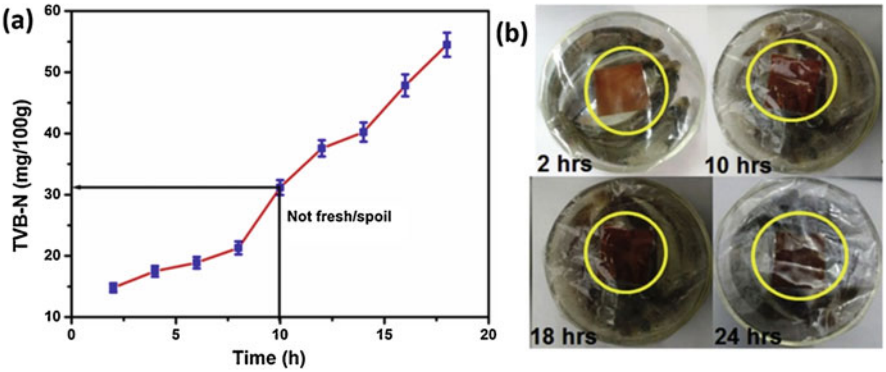


Fig. 19.6 (**a**) The change of TVB-N level of Indian oil sardine fish at room temperature. (**b**) Application of sensing films as sensors for Indian oil sardine fish freshness (Reproduced from Vadivel et al. 2019, Copyright 2020, with permission from Elsevier)

detect the decay of Indian oil sardine fish freshness by measuring the total volatile basic nitrogen (TVB-N). The starting color of the hybrid composite films was yellow (Fig. 19.6), but after 10 h, it changed to orange red due to the rise in TVB-N concentration.

As Fig. 19.6 presents, the hybrid composite films based on CH, PVA, xylan, nHA, and CUM have great potential as an intelligent packaging to determine the spoilage of fish through direct visual inspection. The characteristics of cheeses produced with Lactobacillus helveticus containing high-coliform counts and coated with turmeric and NaALG were recently evaluated (Olivo et al. 2020). The coated cheeses had higher microbial growth although the total coliform bacteria were reduced according to the storage time. In addition, the coverage significantly altered the hardness, gumminess, chewiness, and cohesiveness over time, while the water steam permeability and elasticity were not affected (Olivo et al. 2020). The negative attribute on color, taste, flavor, and nutritional value of the fruit resulting from enzymatic and nonenzymatic browning is a major concern in the food industry (Sarpong et al. 2018). In this respect, the kinetics of enzymatic inactivation and browning pigmentation of apple slices in cold storage have been investigated using three coating gums and distilled water (control). Results from enzymatic inactivation show that gum coatings significantly inhibited the PPO, POD, and AAO activities (Sarpong et al. 2018), especially during the early days of storage when compared with control. XG-based coatings preserved the integrity of tissues of apple better than Acacia and Karaya gums in terms of ΔE and firmness, which indicates their promising application in preserving the color of stored fruit.

The physicochemical features of fresh-cut red apples were successfully preserved during 21 days of storage using α-tocopherol-loaded XG coatings (Galindo-Pérez et al. 2015). It should be pointed out that the polymeric coatings contributed significantly to the lowering of the initial respiration rates. The possibility of replacing the synthetic wet strength resin in paper-making production, with CH of MMW, has been explored by Rollini et al. (2017). The combination of CH, propolis, and microfibrillated cellulose has succeeded in preserving an adequate quantity of active polyphenols in paper manufacturing which could be responsible for antimicrobial activity against *L. innocua* (Rollini et al. 2017). A plethora of studies have shown the applicability of anthocyanins (ACs) as a pH-sensing substance due to the change of their structure as a function of medium pH. ACs have been added to starch (Golasz et al. 2013), CH (Yoshida et al. 2014; Zhang et al. 2014; Halász and Csóka 2018), CH-pectin polyelectrolyte complex (Maciel et al. 2015), and CH/PVA blend (Pereira et al. 2015) and absorbed in bacterial cellulose nanofibers (Pourjavaher et al. 2017) to prepare polysaccharide-based food spoilage indicators. To prevent their dissolving when pH is lowered, the cross-linking of the polymer matrix has been proposed (Maciel et al. 2015; Pereira et al. 2015; Yoshida et al. 2014). Yoshida et al. (2014) showed that AC extracted from grape lowered the solubility of CH and was used as an indicator dye. Halász and Csóka (2018) investigated the effect of black chokeberry pomace extract on the solubility and water uptake properties of CH in a wide range of pH values from 2 to 9. The use of the berry pomace extract as pH indicator to obtain smart CH labels has been also exploited. After evaporation of the solvent and formation of the films, the red solution of CH extract turned into purplish blue-colored film due to the formation of the quinonoidal anhydrobase. The response of the intelligent CH labels incorporating berry pomace extract to pH change is depicted in Fig. 19.7.

L* values were lower for the films with higher ACs content. a* values were higher only in acidic medium (pH of 1–3) (Fig. 19.7). In acidic media, the red color of the CH films was attributed to the flavylium cation structure of ACs (Halász and Csóka 2018), whereas at around pH of 4, the colorless carbinol base form starts to be predominant (Schwarz and Winterhalter 2003). At pH 5 and pH 6, the CH extract films became pale brownish due to the formation of carbinol pseudo base and

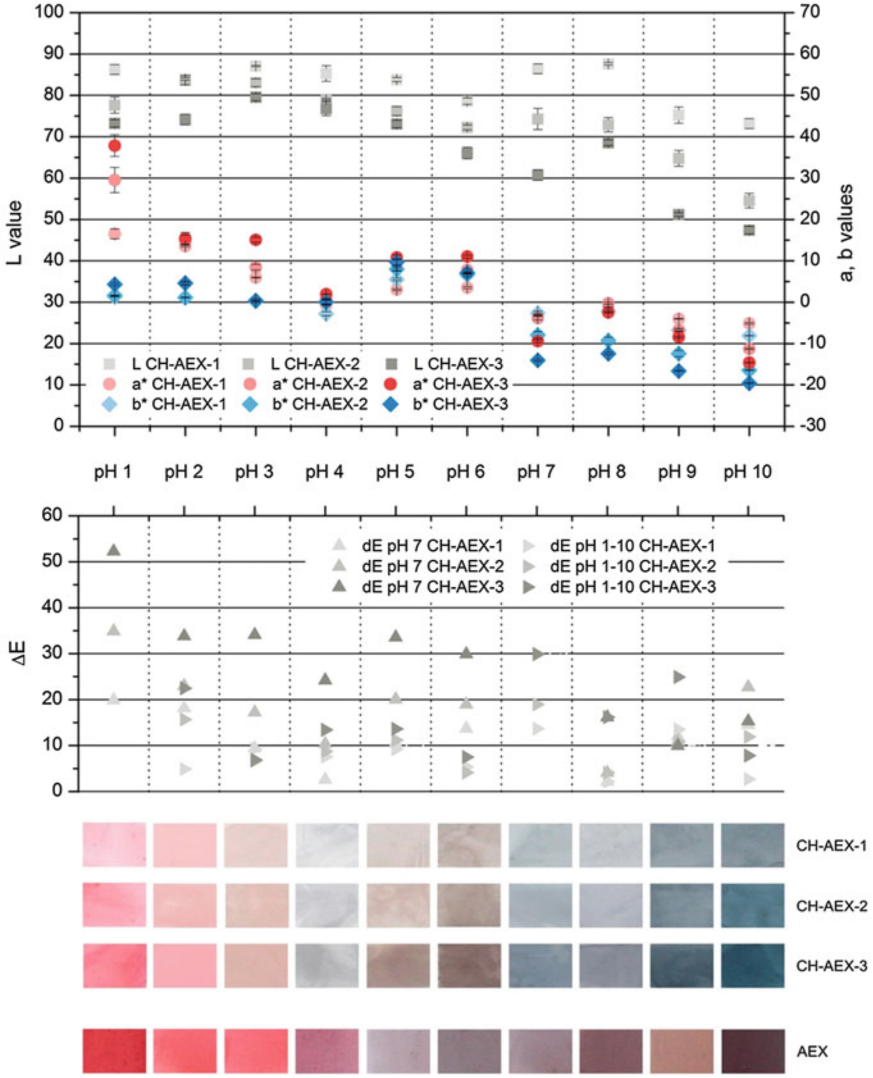


Fig. 19.7 Chitosan-black chokeberry pomace extracts (CH-AEX) films as colorimetric pH indicators. Effects of AEX content and pH onto the color response of the indicator films to pH changes (pH 1–10) determined by the CIE Lab method (lightness, L*; redness or greenness, a*; yellowness or blueness, b*) (Reproduced from Halász and Csóka (2018), Copyright 2018, with permission from Elsevier)

chalcone. At pH ≥ 7, quinoidal anhydrobase appeared, and blue color was easily seen (Halász and Csóka 2018). The greater the berry pomace extract content in CH films, the higher the ΔE became (Fig. 19.7). Since ACs are more stable in acidic media, the CH-based films would be more appropriate for the detection of food degradation correlated with acidic pH change.

19.5 Conclusions

Polysaccharides are ideal candidates to obtain films, membranes, hydrogels, particles, or carrier capsules of the biologic active compounds due to their nontoxic behavior, biocompatibility, and biodegradation capacity at the contact with biological fluids. Among all polysaccharides, chitosan is the most investigated and was proven to possess immense potential as an antimicrobial packaging material owing to its antimicrobial activity and nontoxicity. The use of these polysaccharide-based films offers new opportunities to develop novel smart and biodegradable packaging and solving the daily life issue associated with waste accumulation caused by the intensive use of petroleum-based plastics. The water vapor permeability, the flexibility, the toughness, and the mechanical strength of the polysaccharides-based films have been improved by several approaches including cross-linking and blending with other natural or synthetic polymers. In addition, by incorporation of antioxidant agents, for example, phenolic compounds, films or coatings with enhanced antioxidant and antimicrobial activities have been obtained. Many researchers demonstrated that polysaccharide-based materials can protect food against contamination and microbial spoilage. Polysaccharide-based coatings were also demonstrated to maintain a high product quality during the storage period and to extend the shelf life of food products, which indicate them as promising alternatives to synthetic-based plastics in food packaging applications.

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An Overview on Feasible Production of Bioplastic Polyhydroxyalkanoate (PHA) in Transgenic Plants

20

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Abstract

With the advancements made in the field of plant biotechnology, not only plant traits such as yield, stress tolerance, and nutritional value have been improved but also novel products like edible vaccines, biopharmaceuticals, and even bioplastic have been developed. The first transgenic *Arabidopsis thaliana* plant was genetically engineered by transforming PHA genes from *Alcaligenes eutrophus* to biosynthesize polyhydroxyalkanoate (PHA). PHA is a renewable, biodegradable, and environmentally friendly bioplastic providing an alternative to reduce the toxic effects of conventional plastics on environmental and human health. Production of bioplastic from transgenic plants is a cheaper and safer option than bacterial synthesis because in this method, bioplastics are directly produced from CO_2, salts, water, and solar energy without altering the quality of bioplastic. The successful production of bioplastic especially the PHA from transgenic plants depends on factors such as designing of an effective transgene vector, selection of superior transformation strategy, and higher expression of transgene without negative influence on plant growth and metabolism. To date, various plant species including *Arabidopsis thaliana*, *Nicotiana tabacum,* and *Saccharum officinarum* have been successfully transformed for the production of PHA. But still some crucial factors such as high accumulation of biopolymer, development of a

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reliable and simple downstream method, and ecological safety measures need to be addressed for scale-up of these transgenic plants as bioreactors for efficient production of bioplastic. In this book chapter, biosynthesis pathway of PHA in bacteria, its subcellular compartmentalization in transgenic plants, and rationale behind the biosynthesis of PHA in transgenic plants have been reviewed critically.

Keywords

Bioplastic · Transgenic plants · Biosynthesis · Polyhydroxyalkanoate (PHA) · Polyhydroxybutyrate (PHB)

20.1 Introduction

Advances made in the field of plant molecular biology have allowed the expression of foreign genes in a variety of plants to produce transgenic plants (Rao et al. 2009). Gene transformation in plants was first described in tobacco in 1984. Since then, developments in transformation technology have led to enormous genetic manipulation in plant genes (Narusaka et al. 2012). Genetic engineering has opened up new avenues to use plants for the production of a variety of products. The ability of plants to flexibly express foreign proteins has generated interest in exploring the possibility to synthesize various types of bioplastic in plants (Arai et al. 2004). Due to the durability, inexpensiveness, light weight, and many other applications of bioplastic, it has become an omnipresent material on Earth (Emadian et al. 2017). The interest in producing biodegradable plastic in plants has increased due to the growing awareness of the negative impacts of conventional plastics.

Plastic waste disposal is a big challenge faced globally due to the shrinkage of landfill capacity (Moharir and Kumar 2019). Furthermore, when plastic is dumped into landfills, disintegrated by-products contaminate soil and underground water which is persistent for a long time (Teuten et al. 2009). Alternative to landfills is incineration of plastic, but there are also growing concerns over the toxic emissions during this process (Nagy and Kuti 2016). Another environmental problem associated with plastics is their accumulation in oceans (Cózar et al. 2014). Persistence of plastic at sea leads to its engulfment by marine life as well as their death due to entanglement in plastic debris (Lebreton et al. 2017). Moreover, ingested plastic by marine fauna is transferred to other animals and humans through food chain (Eriksen et al. 2014). Plastic pollution results in creating an environment which promotes breeding of mosquitos and other disease-causing vectors (Okogun et al. 2005). Human health is also being influenced by plastic pollution like a slow poison. Plastic additives are carcinogens and endocrine disruptors, which bioaccumulates through food chain and causing a major health concern (Halden 2010). Additionally, conventional plastic is manufactured from nonrenewable fossil fuel which is depleting and becoming economical challenge. All the aforementioned environmental, social, economic, and health concerns regarding plastic have raised a cry to find alternative, eco-friendly, and sustainable material. Bioplastic is one such material defined as a biopolymer which involves living organisms in its synthesis (Queiroz and Collares-Queiroz 2009). Bioplastic provides several benefits like lower carbon footprint, low cost of manufacturing, less waste buildup, biodegradability, and renewability (Chen 2014). With growing consumer awareness and demand for sustainable products, bioplastic market share accounted for 1% of the plastic market in 2017, which is expected to rise by 40% in 2030. Currently, Europe and Asia are the major bioplastic producers (Zhao et al. 2020). Bioplastic applications in packaging, construction, electronics, medical, automotive, agriculture, and textile industries are also increasing day by day (Peelman et al. 2013; Rusu and Boyer 2011; Liu et al. 2019).

Bioplastics are basically biopolymers which accumulate in the form of intracellular granules in microbes as carbon reserves for energy (Rudin and Choi 2013). The widely known bioplastics include polylactic acid (Narusaka et al. 2012), starch-based plastic, polyhydroxyalkanoate (PHA), cellulose esters, bio-polyethylene (PE), polypropylene, and polyamide (Emadian et al. 2017). Among all types of bioplastics, PHA is the well-studied bioplastic due to its high biodegradability and biocompatibility (Amaro et al. 2019). PHAs are basically polyesters which are synthesized by a vast variety of bacteria for energy storage in the form of lipid inclusion granules within the cell (Poli et al. 2011). In 1926, French scientist Lemoigne discovered PHA for the first time in the form of polyhydroxybutyrate (PHB) in *Bacillus megaterium* (Mohapatra et al. 2017). Some bacteria produces PHA either under nutrients limitation or during growth phase without any nutrient limitation (Muhammadi et al. 2015). Although bacterial plastic or PHAs have distinctive advantage over petro-plastic, their high cost of production is a major limiting factor. Factors such as running cost of fermentation, carbon source, process productivity, and downstream processing contribute to the overall cost of production. Carbon source alone accounts for 50% of total production cost in bacteria (Raza et al. 2018). Therefore, PHA production in transgenic plants on large scale and at a comparable cost has led to the emergence of a new field in PHA research focused on to produce bioplastic in plants (Poirier et al. 1995). Plants are ideal candidates for the production of PHA as compared to bacteria because they are efficient producers of biomass. Furthermore, plants produce foreign proteins, which are biologically active such as antibodies. Importantly, plants are efficient in using direct carbon source through photosynthesis, thus eliminating the input carbon cost (Yunus et al. 2008). Genes coding for PHB from *Ralstonia eutropha* have been transformed into number of plants to produce PHB bioplastic (Menzel et al. 2003). A variety of plant species have been transformed including *Arabidopsis thaliana*, *Nicotiana tabacum, Brassica napus, Gossypium hirsutum,* and *Linum usitatissimum* for the production of PHA (Fig. 20.1) (Ahuja 2014). Continued progress in the development of bioplastic like PHA in transgenic plants will be based on detailed understanding of the pathways involved in microbial PHA biosynthesis and plant metabolism. This chapter provides an overview of the bioplastic production in transgenic plants with particular focus on the bacterial metabolic pathway of PHA along with its transformation and localization in different plants. Furthermore, major hurdles in the

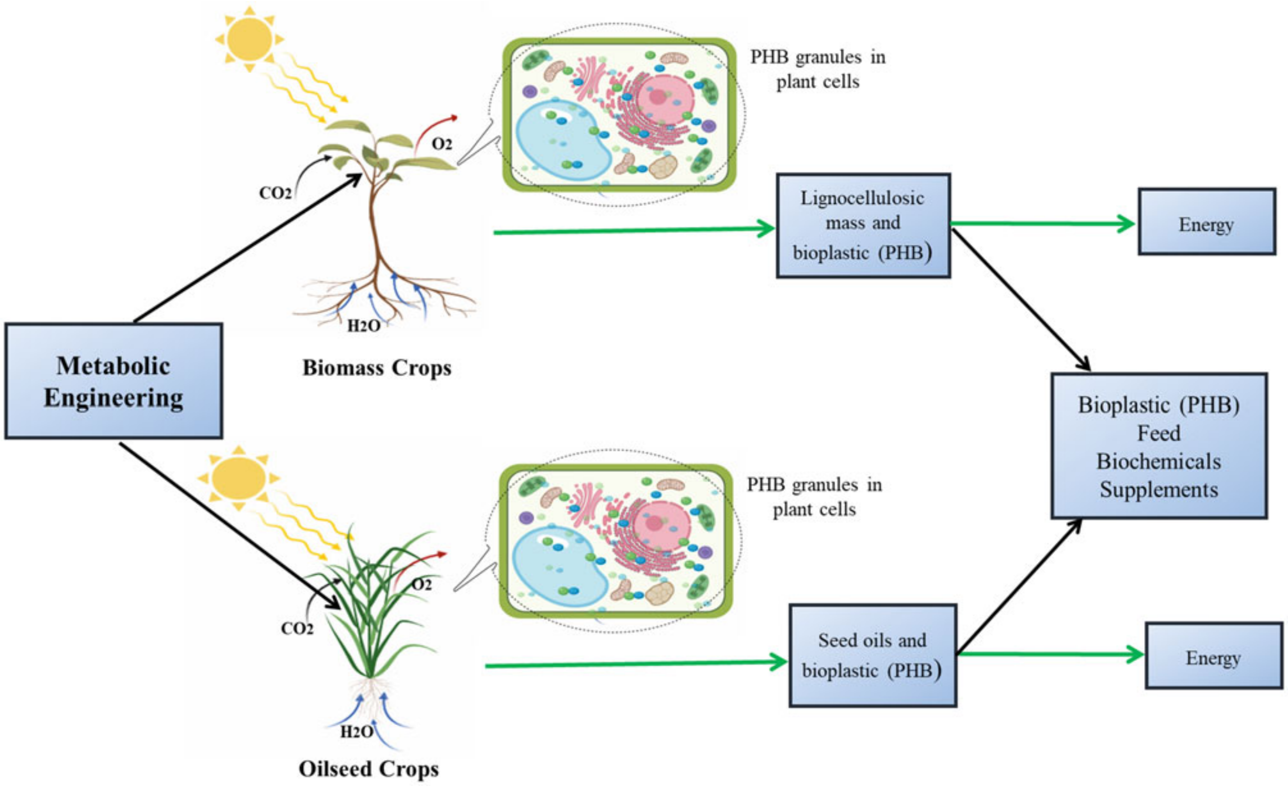


Fig. 20.1 Graphical representation of bioplastic (PHA) productions in different plants by using metabolic engineering

production of bioplastic in transgenic plants and their future perspectives are also discussed critically.

20.2 Biosynthesis Pathway of PHA in Bacteria

PHAs are polyesters which belong to a broad family of microbial storage biopolymers. PHAs subunit is formed by a large group of 3-(R) hydroxy-fatty acids joined together by an ester bond between a hydroxyl group and carboxyl group of adjacent monomer (Lenz and Marchessault 2005). PHAs can be characterized by degree of unsaturation, type of functional group, and chain length. PHAs are mainly divided into three groups based on the number of carbon atoms in monomer units: short-chain length (SCL) consists of 3–5 carbon atoms, medium-chain length (MCL) consists of 6–14 carbon atoms, and long-chain length (LCL) consists of 17 and 18 carbon atoms (Reddy et al. 2003). PHAs production in bacterial cell occurs under specific conditions usually under nutrient limitation such as lack of nitrogen, sulfur, potassium, zinc, oxygen, or magnesium. The polymer type being synthesized by bacteria depends on the food substrate availability (Masood et al. 2015). PHAs are round-shaped granules around 0.1–0.2μm in diameter which are accumulated in cytoplasm (Dobrogojski et al. 2018).

The PHA biosynthesis pathway was first characterized as a series of enzymatic reactions. The PHB biosynthesis pathway is regulated by redox state of cytoplasm and intracellular concentration of pyruvate and available acetyl CoA. Low level of acetyl CoA is favorable for activation of enzymes involved in PHA biosynthesis (Madison and Huisman 1999). Biosynthesis of PHB is well characterized in *Ralstonia eutropha* in which there are three key enzymes involved in PHB biosynthesis as shown in Fig. 20.2. These enzymes are *β*-ketothiolase (*phbA)*, acetoacetyl-CoA reductase (*phbB*), and PHA synthase (*phbC*). The *phb* locus of *R. eutropha* comprises three open reading frames located in one operon known as operon-CAB (Houmiel et al. 1999). *β*-ketothiolase is the first enzyme involved in the PHA biosynthesis. This enzyme catalyzes the reversible condensation reaction of two acetyl-CoA moieties resulting in acetoacetyl-CoA formation. The activity of this enzyme is inhibited by higher level of CoA, which diverts toward the biosynthesis of proteins, nucleic acid, or other macromolecules (Budde et al. 2010). In second stage, NADPH-dependent acetoacetyl-CoA reductase reduces the products of ketothiolase reaction to form hydroxyalkanoic acid. The activity of acetoacetyl-CoA reductase enzyme is directly proportional to NADPH/NADH ratio (Reinecke and Steinbüchel 2009). The third key enzyme PHA synthase is involved in polymerizing hydroxyalkanoate monomers to form scl-PHA/PHB chain (Ayub et al. 2006). In *R. eutropha*, PHB typically accumulates up to 80–85% dry weight (DW) when grown under excess carbon supply such as glucose but limited in essential nutrients like phosphate or nitrogen (Poirier 2002). Under carbon limitation, PHB accumulated in bacterial cell is hydrolyzed and mobilized by PHB depolymerase (*phaZ*) and other related enzymes so that it is utilized by host for its growth and

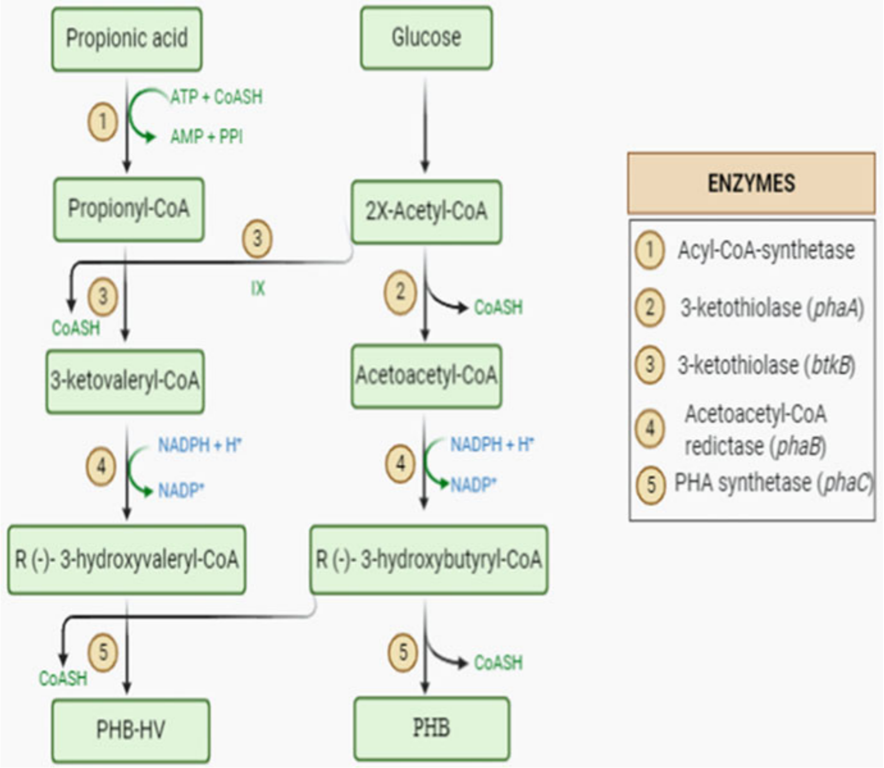


Fig. 20.2 Biosynthesis pathway of poly-(3-hydroxybutyrate) and poly-(3-hydroxybutyrate-Co-3-hydroxyvalerylate) in *Ralstonia eutropha*

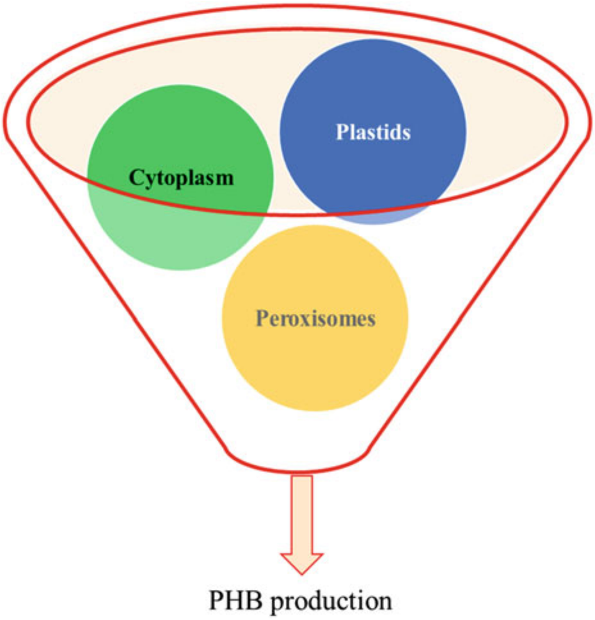
survival. PHB depolymerization results in acetoacetate formation and eventually catabolized to form acetyl-CoA (Mohanan et al. 2020).

Since PHB have relatively poor physical properties, copolymers of PHB have been synthesized with better physical attributes. Copolymers of PHB can be formed by co-feeding of substrates and may lead to the formation of monomers like 3-hydroxyvalerate (3 HV) or 4-hydroxybutyrate (4HB) (Avella et al. 2000). Incorporation of 3-hydroxyvalerate units in PHB results in the formation of copolymer poly-3-hydroxybutyrate-co-3-hydroxyvalerate or PHBV. Adding copolymer leads to lower crystallinity and melting point; hence, PHBV is tougher and less stiff having better commercial uses. Furthermore, addition of glucose and propionic acid in different ratios leads to the production of a variety of PHBV in *R. eutropha* (Wang et al. 2013). In addition to scl-PHAs, mcl-PHAs biosynthesis has also been described. Monomers present in mcl-PHAs may have different functional groups and degree of saturation which can be modified to enhance various structural and physical properties of PHAs. In bacteria, mcl-PHA biosynthesis could be based on either intermediates from β-oxidation of fatty acids/alkanoic acid or products/intermediates of fatty acid biosynthesis (Dobrogojski et al. 2018). The carbon number in mcl-PHA monomers usually depends on the number of carbons in carbon source added to the growth media. For instance, when *Pseudomonas oleovorans* was grown on dodecanoate (C12), it synthesized PHA containing C12, C10, C8, and C6 monomers (Lageveen et al. 1988). Bacteria such as *Ralstonia eutropha, Pseudomonas, Bacillus,* and recombinant *Escherichia coli* have different capability to synthesize PHAs (Poblete-Castro et al. 2014). Different strategies such as supplying renewable carbon source, genetic engineering, or improving precursor supply have been adopted to enhance PHA production in bacteria. However, the high cost of producing PHAs through bacterial fermentation is still a major challenge for commercial use of PHAs (Liu et al. 2016).

20.3 Subcellular Compartmentalization of PHA in Transgenic Plants

In plant, biochemical pathways are compartmentalized, and individual steps of a particular pathway are distributed over different compartments. Metabolic engineering of various pathways involved in biosynthesis of a specific product and its subsequent cellular compartmentalization is a big challenge in plants. Several factors which should be taken into consideration with respect to compartmentalization of a product include (a) the availability of a precursor or substrate in a particular compartment, (b) targeting of foreign enzymes to the specific organelle, and (c) lastly, the application of multigene metabolic engineering (Heinig et al. 2013). Biosynthesis of PHA in plants is feasible due to the availability of primary substrate acetyl-CoA from tricarboxylic acid cycle and fatty acids metabolism. PHA synthesis in plants has been achieved in cytosol, mitochondrion, peroxisome, and plastids due to the presence of acetyl-CoA in all of these subcellular compartments (Dobrogojski et al. 2018). The first attempt to biosynthesize PHA in plants was targeted at cytosol in *A. thaliana* due to the inherent fatty acid α/β oxidation pathway. Therefore, one of three key PHA enzymes, *β*-ketothiolase was already available, and only two enzymes were engineered. Despite the successful production of PHA in transgenic plant was achieved, it was at a low level and compromising the normal health of *A. thaliana* (Poirier et al. 1992). Plastid transformation can provide an alternative approach to cytoplasmic targeting as it allows high-level accumulation of foreign proteins due to polyploid nature of plastids (Ruhlman et al. 2010). Furthermore, multigenes can be stacked together in an operon and co-regulated by transcription from same promoter as done previously in sugarcane, achieving a considerable level of yield (Anderson et al. 2011). Another significance of plastid-targeted expression is that it prevents pleiotropic effects caused by toxicity of transgene products or their interference with other pathways (Fuentes et al. 2018). As chloroplast is a site of fatty acid synthesis with high influx of acetyl-CoA, numerous studies have shown plastid to be the best site for PHA synthesis (Hölzl and Dörmann 2019). PHA synthesis was also targeted to plant peroxisomes by exploiting carbon flux through β-oxidation of fatty acids. Peroxisome of PHA-producing plants significantly enlarged which reflects the capability of these organelles to increase their size in

Fig. 20.3 Different organelles of plants that serve as localization compartments for production of PHA



order to accumulate more amount of PHA (Mittendorf et al. 1998). Peroxisomes provide a promising target as for the synthesis of scl-PHA experimented in sugarcane due to exceptional level of yield (Fig. 20.3) (Tilbrook et al. 2011).

As per the literature reports, among different subcellular compartments, chloroplasts are mostly taken into consideration due to greater influx of acetyl-CoA as well as higher yield without altering plant phenotype. On the contrary, cytoplasm is least reported due to insufficient yield and negative impact on plant traits. Now, there has been a shift toward peroxisomes (for PHA synthesis) due to the constant supply of acetyl-CoA that are suitable for scl/mcl PHAs.

20.3.1 Production of PHA by Transgenic Plants

Different transgenic plants developed for the production of PHA have been discussed here and summarized in Table 20.1.

20.3.1.1 *Arabidopsis thaliana*

Arabidopsis thaliana serves as a model plant in plant molecular genetics, biochemistry, physiology, and development (Meyerowitz 1987). Therefore, Poirier and colleagues first attempted the production of PHB in *A. thaliana*. They introduced only acetoacetyl-CoA reductase (*phbB*) and PHB synthase (*phbC*) from *Alcaligenes eutrophus* into *A. thaliana* through *Agrobacterium tumefaciens* containing Ti plasmid pBI121 transformation. 3-ketothiolase involved in biosynthesis of PHB was not transformed because this enzyme is already found in the cytoplasm of higher plants

Table 20.1 PHAs biosynthesized in different transgenic plants

Plant species	Plasmid	Promoter	Subcellular compartmentalization	Bioplastic type	Yield	Negative impact on plant	Reference
Arabidopsis thaliana	Ti plasmid pBI121	CaMV 35S	Nucleus, vacuole, and cytoplasm	PHB	0.01% FW	Reduction in growth and seed production	Poirier et al. (1992)
Arabidopsis thaliana	Ti plasmid pBI121	CaMV 35S	Plastids	PHB	14% DW	No negative impact observed	Nawrath et al. (1994)
Arabidopsis thaliana and *Brassica napus*	pMON25668	35S	Plastids	PHBV	1.6% DW	–	Slater et al. (1999)
A. thaliana	pART27	CaMV 35S	Peroxisome	MCL PHA (C6–C16)	4 mg/g DW	–	Mittendorf et al. (1998)
Arabidopsis thaliana	pBI ABC	CaMV35S	Chloroplasts	PHB	40% DW	Side effects on plant development and metabolism	Bohmert et al. (2000)
Arabidopsis thaliana	pBM 225	CaMV 35S	Peroxisome	SCL PHA	64.1μg/g DW	–	Arai et al. (2002)
Arabidopsis thaliana	Construct 3I	e35S	Chloroplasts	PHB	14.3% DW	Stunted growth and chlorotic leaves after the application of inducing agent	Kourtz et al. (2007)

(continued)

Table 20.1 (continued)

Plant species	Plasmid	Promoter	Subcellular compartmentalization	Bioplastic type	Yield	Negative impact on plant	Reference
Arabidopsis thaliana	pCAM	35S-C4PPDK	Chloroplast	PHB	5.1–6.9% dwt	None	Kourtz et al. (2005)
A. thaliana	pCAMBIA 1300	35S	Peroxisome	MCL-PHA (C6 to C16)	0.1% DW	–	Moire et al. (2004)
Arabidopsis thaliana	pBM228s	CaMV 35S	Peroxisome	SCL/MCL PHA	220μg/g cell DW	–	Matsumoto et al. (2006)
A. thaliana	–	–	–	P(3HB-co-3 HV)	6.1 mg/g cell DW	–	Matsumoto et al. (2005)
A. thaliana	pBIC1ABWT, pBIC1ABSTQK, and pBIHC1AB	35S	Plastid	SCL/MCL PHA (C4–C14)	Tenfold increase in PHA than wild	–	Matsumoto et al. (2009)
Beta vulgaris	pBI ABC	CaMV 35S	Leucoplast	PHB	55 mg/g DW	–	Menzel et al. (2003)
Black Mexican sweet maize	pACE2, pACE3, and pACE4	–	Peroxisomes	PHB	2 mg/g FW	–	Hahn et al. (1999)
Brassica napus	pMON36814	*Lesquerella* hydroxylase promoter (P-Lh)	Leucoplasts	PHB	7.7% FW of mature seeds	–	Houmiel et al. (1999)
Camelina sativa	–	–	Plastids	PHB	19.9% DW	–	Patterson et al. (2015)
Camelina sativa	–	–	Plastids	PHB	15% DW	–	Malik et al. (2014)
Elaeis guineensis	pMS29 and pMS31	MSP1	Plastid	PHB and PHBV	–	–	Omar et al. (2008)

Elaeis guineensis	pME22 and pME26	Maize ubiquitin promoter	Plastid	PHB and PHBV	–	–	Ghulam Kadir et al. (2008)
Elaeis guineensis	pMS29 and 35SpEGFP	MSP1	Mesocarp	PHB	–	–	Omidvar et al. (2008)
Elaeis guineensis	pRMIN and pLMIN	CaMV 35S	Plastids	PHBV	–	–	Ariffin et al. (2011)
Elaeis guineensis	pJLPHB3	CaMV 35S	Plastid	PHB	6.06-fold higher than non-transformed plants	–	Ismail et al. (2010)
Elaeis guineensis	pFA2 and pFA3	CaMV 35S	–	PHV	–	–	Ahmad Fuad et al. (2008)
Elaeis guineensis	pME22	–	Mesocarp	PHB	0.043% dwt	No negative effects	Parveez et al. (2015)
Glycine max	p1300t-PHB	Ep 1.5 promoter	Seed coat	PHB	0.36% DW	Nil	Schnell et al. (2012)
Gossypium hirsutum	pFbL2A-B and p35-C	E6 cotton promoter	Cytoplasm	PHB	30 to 3440μg/gm of dry fiber	Nil	John and Keller (1996)
Linum usitatissimum	Single BinAR and triple BinHyg constructs	14–3-3 and CaMV 35S	Chloroplasts	PHB	70-fold higher PHB produced than wild type	Decrease in starch content	Wróbel et al. (2004)
Linum usitatissimum	pBinARHygABC	–	Plastids	PHB	4.62 lg/g FW in M50 line	–	Szopa et al. 2009
Linum usitatissimum	pBinARHygABC 14–3-3	CaMV 35S	Plastids	PHB	3.80μg/g FW	Decrease in glucose, starch, fatty acid, and citric acid as well as lignin levels	Wróbel-Kwiatkowska et al. (2007)

(continued)

Table 20.1 (continued)

Plant species	Plasmid	Promoter	Subcellular compartmentalization	Bioplastic type	Yield	Negative impact on plant	Reference
Linum usitatissimum	pART27	CaMV 35S	Peroxisome	mcl-PHB	1.6 mg/g DW	–	Wróbel-Kwiatkowska et al. (2019)
Medicago sativa L.	pMON25948 and pMON25949	CaMV 35S	Chloroplasts	PHB	0.025 to 1.8 g kg^1 DW of leaves	No negative impact on growth and fertility	Saruul et al. (2002)
Nicotiana tabacum	pBM205E	CaMV 35S	Plastids	PHB	0.001% DW	Reduction in plant growth	Nakashita et al. (1999)
Nicotiana tabacum	pBMR205	CaMV 35S	Plastids	PHB	0.09% DW	None observed	Arai et al. (2001)
Nicotiana tabacum	pTRV-PHB	Prrn and PpsbA	Chloroplast	PHB	100-fold increase	None observed	Zhang et al. (2002)
Nicotiana tabacum	pCAB, pCA, and pUCaadA	–	Plastids	PHB	15–18% DW	Delayed flowering	Bohmert-Tatarev et al. (2011)
Nicotiana tabacum	pKCZ	psbA promoter	Plastids	PHB	1.7% DW	Growth deficiency and male sterility	Lössl et al. (2003)
Nicotiana tabacum	pPT06	Plastid rRNA operon	Plastids	PHB	50–160 ppm of total DW	–	Arai et al. (2004)
Nicotiana tabacum	pKCZ	T7RNAP promoter	Plastids	PHB	1383 ppm in DW	–	Lössl et al. (2005)
Nicotiana tabacum	pBIwBwC, pBIwBeC, pBIeBeC, and pBIwCeC	CaMV 35S	–	PHB	211µg/g DW in eBeC transformants	–	Matsumoto et al. (2011)

Oryza sativa L. var. Nipponbare and *Tamarix* sp.	pIG221 and pIG121Hm	rbcS	Cytoplasm	PHB	0.5% DW in rice and 0.32% DW in Tamarix	–	Endo et al. (2006)
Panicum virgatum	pMBXS155 and pMBXS159.	*cab-m5* light-inducible and rubi2 promoter	Chloroplasts	PHB	3.72% dry weight of PHB in leaf tissues	None observed	Somleva et al. (2008)
Populus tremula × alba	pCAM	Glucocorticoid receptor response element minimal promoter	Chloroplasts	PHB	1–2% DW	10–34% decrease in height, diameter, and total mass above 1% PHB accumulation	Dalton et al. (2011)
Ralstonia eutropha	SE100	TYLCV IR promoter and phaCAB operon	Chloroplast	PHB	–	–	Mozes-Koch et al. (2017)
Saccharum sp.	pEMU-Kn	pEmu	Cytosol, mitochondria, and plastid	PHB	1.88% DW in plastids	–	Petrasovits et al. (2007)
Saccharum sp.	–	–	Plastids	PHB	1.77% of leaf DW	None	Purnell et al. (2007)
Saccharum sp.	pJ2K	Maize *ubi*-1 promoter	Peroxisome	SCL/MCL PHA (C4–C16)	0.015% DW	–	Anderson et al. (2011)
Saccharum sp. and *Arabidopsis thaliana*	pGreenII0229-C6	CaMV 35S	Peroxisome	PHB and PHBV	1.6%–1.8% DW/ leaves	–	Tilbrook et al. (2011)

(continued)

Table 20.1 (continued)

Plant species	Plasmid	Promoter	Subcellular compartmentalization	Bioplastic type	Yield	Negative impact on plant	Reference
Saccharum sp.	pMBXS165, pMBXS166, and pMBXS167	Cv, *ubi2, rubi2,* and *cab-m5*	Plastid	PHB	2.74% DW controlled by *cab-m5*	None	Petrasovits et al. (2012)
Transgenic *Saccharum* sp.	–	–	Plastid	PHB	50–60% yield/plant biomass upon using class A herbicide	–	Petrasovits et al. (2013)
Transgenic *Saccharum* sp.	–	–	Seedlings	PHB	1.8% DW PHB in citrate synthase knockdown line	–	Tilbrook et al. (2014)
Saccharum sp.	pCab-TP-NphT7	Cab-m5	Chloroplast	PHB	11.8% DW of leaves	–	McQualter et al. (2015)
Solanum tuberosum	pAPP63	E-35S promoter	Cytoplasm	Polyhydroxyoctaonate	0.022–9.7 mg/g DW	None	Romano et al. (2003)
Solanum tuberosum	pAPP100 and pAPP101	E-35S	Plastids	mcl-PHAs (C-6 to C-12)	0.03% DW	–	Romano et al. (2005)
Zea mays	pPHBA, pPHBB, pPHBC, and pBY520 or pJS 101	–	Chloroplast	PHB	–	–	Zhong et al. (2003)

involved in mevalonate synthesis. PHB accumulated in cytoplasm, nucleus, and vacuoles with significant reduction in growth and seed production. PHB yield was as low as 0.01% fresh weight (FW) which could be due to the depletion of cytoplasmic acetyl-CoA pool. Despite little success in high yield of PHB in plants, this attempt paved way for future PHA production in transgenic plants (Poirier et al. 1992). Due to little success with cytoplasmic synthesis of PHB, feasibility of plastid-localized PHB production was tested in *A. thaliana*. Flux of acetyl-CoA is high in plastids as they are the main site of fatty acid synthesis and lipid storage. This strategy leads to 100-fold increase in PHB production without any deleterious effects on plant metabolism and sterility (Nawrath et al. 1994).

Further experiments showed the possibility of producing PHBV in plastids of *A. thaliana* and *Brassica*. Four genes *IlvA, bktB, phbB,* and *phbC* were transformed into plants via *A. tumefaciens*-mediated transformation. *IlvA* encoded for the conversion of threonine to 2-ketobutyrate to generate propionyl CoA. Once propionyl CoA was generated, its co-substrate acetyl-CoA were converted to D-3-hydroxyvaleryl-CoA by the expression of *BktB* and *PhbB*, which was polymerized into PHBV by the action of *phbC* (Slater et al. 1999). It is a novel strategy in which all three genes necessary for PHB biosynthesis were combined in single plasmid and transformed *A. thaliana* in a single transformation event, unlike the previous researches in which all three genes were transformed into plants individually and later combined via crossing. The results showed surprisingly high level of PHB accumulation as high as 40% of DW in leaf chloroplast (Bohmert et al. 2000). Similarly, a single gene construct encoding thiolase and reductase was developed since both these enzymes function catalytically as homotetramers. However, the yield of PHB as a result of fusion proteins reduced in *A. thaliana* which could be due to problems related with proper folding of enzymes (Kourtz et al. 2005). When mutated 3-ketoacyl-acyl carrier protein (ACP) synthase III (*FabH*) and mutated PHA synthase (*PhaC1*) genes from *E. coli* and *Pseudomonas* along with PhaA and PhaB genes were introduced into *A. thaliana*, it resulted in a tenfold greater production of PHA. Furthermore, *FabH* and PHA synthesis genes facilitated in the synthesis of scl/mcl PHA in plastids (Matsumoto et al. 2009). Another strategy to enhance PHB production without compromising plant health includes inducible promoter system. By delaying the expression of transgenes with the help of externally applied inducing agent would promote the expression of transgene at later developmental stage of plant. With the application of ecdysone to engineered *A. thaliana*, up to 14.3% increase in PHB production per DW was observed in young leaves of T_2 progeny (Kourtz et al. 2007). Similarly, PHA synthase gene isolated from *Aeromonas caviae* was mutated by in vitro evolutionary techniques. The mutated gene when introduced into *A. thaliana* led to eight- to tenfold increase in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3 HV)] accumulation than wild-type PHA synthase. This suggests that in vitro-mutated PHA synthase is efficient in enhancing productivity of PHA and regulating monomers of PHA (Matsumoto et al. 2005).

20.3.1.2 *Elaeis guineensis* and *Elaeis oleifera*

Oil palm (*Elaeis guineensis*) is an agro-economically important crop due to its ability to produce large amount of oil in fruit mesocarp. Acetyl-CoA carboxylase is a key enzyme involved in fatty acid synthesis and possesses high acetyl-CoA pool (Lal 2018). Downregulating acetyl-CoA carboxylase along with the introduction of PHA biosynthesis genes could maximize PHA synthesis in oil palm (Omar et al. 2008). The embryogenic calli of oil palm were transformed through biolistic bombardment of DNA precipitated onto gold micro-carriers. The plasmid pME22 contained *phaC, bktB,* and *phaB* genes for the synthesis of PHB from *Alcaligenes eutrophus,* whereas an additional gene encoding for threonine dehydratase *tdcB* from *E. coli* was incorporated into pME26 for PHBV synthesis. The genes were driven by maize ubiquitin promoter, which led to stable transformation of oil palm (Ghulam Kadir et al. 2008). Similarly, genetic transformation of oil palm calli and plantlets with pJLPHB3 vector driven by CaMV 35S showed around sixfold higher expression of PHB genes (Ismail et al. 2010). In another study, micro-projectile bombardment of oil palm embryogenic calli led to the production of 0.043% PHA per DW in mesocarp (Parveez et al. 2015). The use of tissue-specific promoter helps in reducing the negative impact of transgene expression on growth and development. Hence, mesocarp-specific promoter (MSP1) was used to target the expression of PHB in mesocarp (Omidvar et al. 2008). Furthermore, flanking the genes with matrix attachment region from tobacco (RB7MAR) aids in stabilizing transgene expression, enhancing expression level, and reducing gene silencing effects (Mat Yunus et al. 2008). Intensive research in oil palm biotechnology is required to exploit high oil-producing oil palm for bioplastic production.

20.3.1.3 *Linum usitatissimum*

Genetic engineering of flax for the production of PHB has been carried out in order to improve the quality of flax fibers but not for bioplastic production. The cotyledons and hypocotyls of flax were transformed with PHB genes through *A. tumefaciens*-mediated transformation. Addition of 14–3-3-specific promoter expressed *phbA* gene in stem. The PHB synthesis improved flax fiber mechanical strength in stem significantly (Wróbel et al. 2004). Similarly, substantial improvement in elastic properties, strength, Young's modulus, and phenolic acid content was also observed (Szopa et al. 2009). The importance of improving flax fibers with the addition of PHA is based on its biomedical application. Platelet aggregation is a phenomenon which promotes blood clot formation and can be induced by external devices coming in contact with blood. So materials which induce platelet aggregation are not used as implants (Prasad 2009). The ability of PHA composite flax fiber in inducing blood platelet aggregation was studied, which showed no platelet aggregation (Szopa et al. 2009). The biomechanical properties of flax enhanced with the expression of *phaC1* gene producing mcl-PHA (Wróbel-Kwiatkowska et al. 2019).

20.3.1.4 *Nicotiana tabacum*

In order to produce bioplastic in transgenic plants, tobacco plant has been successfully transformed several times. The first attempt to produce PHB in tobacco failed to produce any significant amount of PHB in cytoplasm (Nawrath et al. 1995). In second attempt, the tobacco plant was transformed with pBM205E expression vector containing *phbB* and *phaC*$_{AC}$ gene regulated by CaMV 35S promoter. The productivity of polyester was low in cytoplasm indicating low acetyl-CoA influx along with reduction in growth of plant (Nakashita et al. 1999). Later on, plastid-targeting signal peptide was utilized which enhanced 100-fold productivity of PHB in plastids (0.09% DW) as compared to cytoplasm (0.001% DW) (Arai et al. 2001). Chloroplast genetic engineering technology for PHB transgene expression is advantageous over mitochondrial or nuclear DNA transformation and resulted in high level of transgene expression and absence of gene silencing (Adem et al. 2017). Therefore, pTRV chloroplast integration and expression vector were designed containing PHB genes and *aadA* gene inserted between *rpl2-trnH-psbA* and *trnK-ORF509A* plastid sequence for homologous recombination. This strategy successfully transformed PHB genes into tobacco plastids (Zhang et al. 2002).

Metabolic engineering of transgenic tobacco lines BC3 and rCAB8, which produces PHB in cytosol and plastids, respectively, allowed enhanced production of PHB. D-(+)-quizalofop-ethyl was sprayed onto transgenic tobacco lines, which acted as an inhibitor of acetyl-CoA carboxylase (ACCase), increased supply of acetyl-CoA, and activation of PHB biosynthesis pathway. The results showed PHB production was 2.5-fold higher in plastid than cytosol (Suzuki et al. 2002). PHB operon under plastid regulatory elements resulted in accumulation of PHA up to 1.7% DW in tissue culture, whereas in mature greenhouse plant, content of PHB was extremely low, and plants were male sterile. Nonetheless, this report showed better accumulation than previous experiments with tobacco (Lössl et al. 2003). Furthermore, the artificial operon containing bacterial-derived PHB genes with an addition of synthetic ribosome-binding site (RBS) enhanced translation of PHB genes. PHB production when grown on MS media was 50–160 ppm of total DW; however, under greenhouse conditions, productivity reduced to 10–60 ppm of total DW. The decrease in productivity could be due to low photosynthetic activity of plants growing on MS media and higher carbon flux into plastids for biosynthesis of fatty acids (Arai et al. 2004). In order to avoid limitations involved in growth and development of plant due to transgene expression, a trans-activation system was developed to induce *phb* operon expression. In this system, ethanol-inducible T7RNA polymerase (T7RNAP)-targeted plastid *phb* operon controlled by T7 regulatory element was introduced. So when the plants were treated with 5% ethanol, PHB was synthesized at 1383 ppm per DW (Lössl et al. 2005). Further experiments improved productivity level up to 15% to 18% DW using synthetic operon (Bohmert-Tatarev et al. 2011). In another report, codon optimization of *PhaB* gene also effectively increased PHB production (Matsumoto et al. 2011). Hence, by adopting and developing different strategies could help in further enhancement of PHA production in tobacco plants.

20.3.1.5 *Saccharum officinarum*

Sugarcane (*Saccharum officinarum*) can be a good candidate crop for PHB production due to its very large biomass and easy cultivation method. The embryogenic calli of sugarcane were bombarded with pEMU-Kn plasmid to target PHB pathway to cytosol, plastid, or mitochondria. PHB accumulation was detected in leaves of plastid lines at a level 1.88% DW, while trace amount of PHB accumulated in cytosol and no PHB was found in mitochondria (Petrasovits et al. 2007). Further analysis at whole plant level showed increase in PHB accumulation with the age of leaf as well as increasing from the base of leaf to tip of leaf. The maximum PHB accumulation recorded was 1.77% DW of leaf without any compromise to plant growth and development (Purnell et al. 2007). Multigene strategy was applied to produce scl/mcl PHA in peroxisome of sugarcane. The transformation vector consisted of genes encoding for three PHB enzymes and enoyl-CoA hydratase for peroxisomal targeting as well as acyl-ACP thioesterase and 3-ketoacyl-ACP synthase genes targeting plastid to enhance β-oxidation flux to peroxisome. The transgene accumulated 0.015% leaf DW PHB copolymer consisting of C4-C16 3-hydroxyalkanoic acids (Anderson et al. 2011). Peroxisomal PHB production has a good potential to reach commercial level as PHB accumulated at a level of 1.6–1.8% DW in sugarcane leaves (Tilbrook et al. 2011).

Another strategy adopted to enhance the polymer production in transgenic sugarcane was the use of strong promoter. The promoters tested were maize ubiquitin promoter (*ubi1*), rice ubiquitin 2 (*rubi2*), Cavendish banana streak badnavirus promoter (*Cv*), and maize chlorophyll A-/B-binding protein (*cab-m5*). The *Cv* promoter-generated transgenic sugarcane lines showed highest polymer content; however, the transgene expression was transient and diminished after 6 months. The best of four promoters was *cab-m5* accumulating 2.74% DW PHB in primary transgenic sugarcane. The polymer content increased as plants matured for all lines except in *Cv* lines, which means PHB was not metabolized with time (Petrasovits et al. 2012). Chemical inhibition of ACCase by using different herbicides or chemical ripeners is an effective strategy to enhance sugar content in sugarcane (Dalley and Richard 2010). A study revealed that the class A herbicide treatment of sugarcane inhibited ACCase activity, reduced fatty acid biosynthesis, and showed up to 50–60% PHB yield/leaf biomass under greenhouse conditions (Petrasovits et al. 2013). Increasing the substrate availability from β-oxidation by knocking down the activity of citrate synthase for PHB biosynthesis enhanced PHB production. Since peroxisomal acetyl-CoA is converted into citrate by citrate synthase which leads to major efflux of carbon from peroxisomes, artificial micro-RNA (amiRNA) technology was used to reduce the activity of peroxisomal citrate synthase leading to threefold increase in PHB production (1.8% DW) in 10 days old seedlings (Tilbrook et al. 2014). McQualter and colleagues studied the various factors affecting PHB accumulation in mesophyll cells of sugarcane, which suggested the ineffective capturing of substrate from fatty acid biosynthesis (McQualter et al. 2014). Subsequently, they constructed a vector containing codon-optimized DNA fragment *nphT7*, *phaB,* and *phaC* genes transformed in sugarcane, resulting in overall increase in yield of PHB in mesophyll cells. This showed that *nphT7* encoding for acetoacetyl-CoA synthase was more effective in accessing acetyl-CoA than bacterial thiolase (McQualter et al. 2015).

20.3.1.6 Other Plants

There are many other plants besides the above mentioned in which PHA production is possible but less widely studied. For example, the hairy roots of sugar beet (*Beta vulgaris*) were tested for the possibility of PHB production due to its ability to produce the highest biomass. Targeting the leucoplast for PHB synthesis resulted in economically relevant amount in *Brassica napus* at level of 7.7% FW of seed and 55 mg/g DW in *Beta vulgaris* (Houmiel et al. 1999; Menzel et al. 2003). Leucoplasts are colorless plastids involved particularly in long-chain fatty acid biosynthesis and thus have good influx of acetyl-CoA and a suitable target for PHA biosynthesis in roots (Plaxton 2017). *Camelina sativa* plastids have also shown good yield of PHB (19.9% DW) (Patterson et al. 2015). Transformation of PHB genes in cotton also resulted in better thermal properties of cotton fiber and higher heat capacity (John and Keller 1996). Similarly, multigene transformation strategy was adopted to transform alfalfa plants, which resulted in stable expression but limited production of PHB (0.34% DW) (Saruul et al. 2002). Many other crop plants such as soybean, rice, potato, switchgrass, and corn were successfully transformed with PHB genes; however, they showed little success (Schnell et al. 2012; Endo et al. 2006; Romano et al. 2003; Somleva et al. 2008; Zhong et al. 2003). The production of bioplastic in a variety of plants has been well documented, but more research is required to achieve economically favorable yield.

20.4 Conclusions and Future Outlooks

The feasibility of producing PHA in plants at large scale and reasonable cost was first demonstrated in *Arabidopsis thaliana*. After that, a wide variety of plants have been investigated for the production of PHA; however, still there are major challenges to be resolved for successful production of PHA in plants. The main problems included negative impact on plant phenotype, transient transgene expression, low-product yield, poor monomer composition, and hurdles in scaling up. It has been reported that the expression of PHA genes resulted in reduction in plant growth and development as well as low transformation rate in potato and tobacco. This could be due to metabolic burden on plant, deletion of acetyl-CoA pool, or undesirable interaction between PHA intermediates and plant metabolites. To address this issue, use of inducible promoter was a good strategy which enhanced yield of PHB twofold without compromising plant phenotype in *Arabidopsis* (Bohmert et al. 2000). Expression of polycistronic operons using a strong promoter for plastid targeting of PHB could also be helpful.

To make PHA production in transgenic plants cost-effective, high yield is very important. Various strategies have been studied to enhance the polymer productivity. The production of PHA in oil rich crops with high acetyl-CoA influx such as rape seeds would be a better option for bioreactor level production (Houmiel et al. 1999). Targeting other organelles such as peroxisome where fatty acid metabolism is very high represents a logical subcellular compartment for the synthesis of PHA. This strategy has been adopted in several crops such as black Mexican sweet maize and sugarcane for production of considerable yield of PHAs (Hahn et al. 1999; Tilbrook et al. 2011). Another strategy tested was chemical inhibition of enzymes metabolizing acetyl-CoA in transgenic sugarcane to increase the influx of acetyl-CoA toward PHB biosynthesis pathway (Petrasovits et al. 2013). Production of scl/mcl PHAs than PHB is more desirable due to their better properties and commercial uses. Therefore, *PHA*C synthase from *A. caviae* was transformed into *Arabidopsis* to produce scl-PHA C3-C7 copolymer which possesses better qualities (Arai et al. 2002). The composition of PHA monomers can be controlled by inserting genes from other bacteria or adding specific sugars with specific carbon number. Additionally, in vitro mutagenesis of enzymes could enhance the yield as well as can produce desirable copolymer as demonstrated in *A. thaliana* (Matsumoto et al. 2005).

Application of synthetic biology for the conversion of biomass into bioplastic has been experimented in bacteria. A group of scientists were able to construct synthetic pathways in *E. coli* which enhanced the fermentation of sugar to form PHB under anaerobic conditions from 29 to 48% (Pei et al. 2011). Likewise, applying synthetic biology to plants for the production of PHA could be an effective strategy where knowledge from computational biology, biotechnology, and genetic engineering are integrated. The successful transformation and expression of bioplastic in transgenic plants at a good yield is not the only challenge, but downstream processes such as PHA extraction at low cost are also a great hurdle. Although various technologies for large-scale extraction of other bio-products from transgenic plants have been developed, the invention of new methods for PHA extraction without modifying original product is still in infancy (Yang et al. 2020). Furthermore, a detailed study of PHA metabolism in plants and its associated metabolic changes is required for successful industrial translation of transgenic plant research.

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21

Engineering Strategies for Efficient and Sustainable Production of Medium-Chain Length Polyhydroxyalkanoates in Pseudomonads

Elías R. Olivera and José M. Luengo

Abstract

Most bacteria belonging to the genus *Pseudomonas* can accumulate medium-chain poly-3-hydroxy-alkanoates (mcl-PHA) as storage materials. For this reason, these bacteria have a well-preserved enzymatic mechanism which involves the participation of a polymerization system (consisting of two different polymerases, PhaC1 and PhaC2), two proteins needed for the structural organization of the mcl-PHA granules (the phasins PhaI and PhaF); a regulatory protein (PhaD), and finally, a polymer-hydrolyzing enzyme (depolymerase, PhaZ) which releases the monomeric units from the granule. In the first stage, PhaC1 and PhaC2 synthesize the polymer using (*R*)-3-hydroxyacyl-CoA precursors obtained throughout the β-oxidation of longer fatty acids or from acetyl-CoA following the de novo fatty acid biosynthetic pathway. All the polymerases described in *Pseudomonas* have a wide substrate specificity, which allows for the collection of a huge number of PHAs (homopolymers and copolymers), having varied physicochemical characteristics and interesting biotechnological applications. Therefore, when considering the production of PHAs, there are some highly relevant aspects that must be taken into account. Thus, the monomer precursor selection (raw material sources), the precise characterization of their biosynthetic pathways, its metabolic networking, and the manipulations needed to ensure an efficient flow of intermediaries are essential aspects to achieve both a good bacterial growth and a high production of the desired polymer(s). This chapter focuses on describing the different mechanisms by which pseudomonads can generate useful PHA monomers as well as the strategies followed to improve the

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efficiency of these processes. Additionally, different approaches to allow for industrial PHA production will be discussed.

Keywords

Pseudomonas · Plastic materials · Polyhydroxyalkanoates · PHAs · *pha* genes · PHA precursors · Genetic regulation · Industrial production

21.1 Introduction

The massive use of petrochemical plastics started in the 50s to substitute traditional materials such glass, metal, wood, or cotton. Nowadays, our modern everyday life is inconceivable without the use of these types of materials. Many of the items we use continuously are made of some kind of synthetic polymers, most of them based on fossil fuels, which we call plastics. Pliability, stability, durability, low weight, and acid resistance, as well as ease and cost-effectiveness of processing, have been some of the milestones for its massive use in manufacturing industries, ranging from packaging to building and construction technology, electrical and electronic appliances, automobile and aircraft manufacturing, and healthcare industry (Strong 2006; Andrady and Neal 2009). In 2018, global plastics production almost reached 360 million tons, 30% made in China, 18% in the countries belonging to the North American Free Trade Agreement (Canada, USA, and Mexico), and 17% in Europe. Since 2002, the European average annual growth has been estimated as a constant rate of about 1.6% even during the 2007–2008 global economic crisis, although the increase in global production has been estimated at being about 4% (Rhodes 2018; PlasticsEurope 2019). Thus, it has been forecasted that by 2050, the projected amount of plastics could reach about 1124 million tons (Rochman et al. 2013; World Economic Forum 2016).

However, the high-level production, the extensive use, and the rapid disposal have resulted in their large-scale accumulation as waste. Just in the year 2018, 29.1 million tons of plastic postconsumer waste were collected in the European Union, United Kingdom, Norway, and Switzerland, of which 75.1% was recovered through recycling and energy recovery processes, but even so, 24.9% (7.25 million tons) was discharged into the environment, usually disposed in garbage dumping landfills (Geyer et al. 2017; PlasticsEurope 2019). In this way, the ubiquitous presence of plastics both in the terrestrial and aquatic realms suggests that they are a key geological indicator of the Anthropocene as a distinctive stratal component (Zalasiewicz et al. 2016).

On the other hand, some of the characteristics that have made plastics a material of preferred use in our lives are durability and stability and have resulted in a double-edged sword since they imply little or no biodegradability of these materials when they ended up as plastic waste. Although some countries manage the main fractions of their plastic garbage through incineration or recycling in controlled industrial facilities, one of the main problems at a global level is its release in the environment

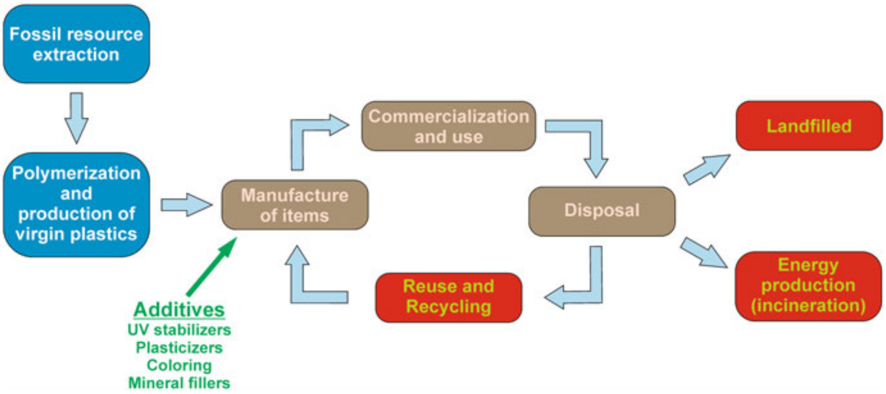

Fig. 21.1 Generic life cycle stages of a petrochemical plastic from fossil extraction resources to end of life as landfilled waste or their incineration for energy recovery

(Fig. 21.1). Spillage of recalcitrant plastic waste causes a colossal impact on the environment, especially in hydrosphere. Thus, 10% of the manufactured plastics enters aquatic environments. From these, 8–13 million tons ended up in the marine habitat through direct spillage or wind transport or in rivers, leading to the large-scale pollution in the oceans, mobilizing public opinion with terms such as “the Great Pacific Garbage Patch” and the “Trash Vortex” (Barnes et al. 2009; Thompson et al. 2009; Cole et al. 2011; Cózar et al. 2014; Jambeck et al. 2015; Li et al. 2016; Horton et al. 2017; Bläsing and Amelung 2018; Hahladakis 2020).

Marine plastic waste is the direct cause of damage and even the death of marine animals and birds, either because they become trapped in them or because they confuse plastic with food (especially microplastics, plastic particles <5 mm in diameter, produced from the breakdown of larger plastic litter items or directly from textiles, medicines, and pellets for plastic production) (Laist 1997; Vlietstra and Parga 2002; Cadée 2002; Wang et al. 2019; Xu et al. 2020; Yong et al. 2020; de la Torre 2020). The wide distribution of microplastics in the food chain, with still scarcely known effects on biodiversity and human health, is an increasingly recurrent topic in the literature, not only in scientific literature but also in general literature (Avio et al. 2017; Wang et al. 2019; Xu et al. 2020; de la Torre 2020). This harmful effect on the environment is further aggravated as plastics contain additives such as plasticizers, UV stabilizers, and mineral fillers (Fig. 21.1). Moreover, during combustion of plastic waste for energy recovery, harmful chemical compounds such as hydrogen chloride, hydrogen cyanide, furans, and dioxins are released. Thus, both during the process of its chemical synthesis and throughout its degradation, molecules are released which are usually very toxic for most living beings (Hahladakis et al. 2018; Campanale et al. 2020). In addition, plastic pellets and microplastics adsorb in their surface-persistent organic pollutants, such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and dichlorodiphenyl trichloroethane, all of which pose an environmental aggression as well as a health risk, since they have a carcinogenic, mutagenic, and/or teratogenic nature (Chua et al. 2014; Xu et al. 2020; Wang et al. 2020).

Another relevant aspect about plastics that must be considered is their own inception on a petrochemical basis produced from fossil oil, gas, and coal. An average of about 4% of annual petroleum production is used as raw material for its conversion into plastics, and together with the energetic contribution for their manufacture, their cost from the petrochemical total production could reach 8% of fossil fuels. According to this, plastic production will account for 20% of global oil consumption by 2050 (World Economic Forum 2016). However, in return, the use of lightweight plastics replacing heavier conventional materials could reduce the cost in fossil fuels in transport applications (Andrady and Neal 2009; Hopewell et al. 2009).

Plastics also contribute to greenhouse gas emissions and climate change at every stage of their life cycle (Fig. 21.1), from production to refining as well as the way they are managed as a waste product. Although material refinement is the part of greatest greenhouse gas production of this life cycle, incineration creates the most CO_2 emissions among the plastic waste management methods. In 2019, CO_2 equivalent generated by production of plastics and incineration of the plastic waste amounted to approximately 860 million tons. Considering the expected growth of plastic usage for 2050, if plastic waste is incinerated in an average amount like the current one, optimistically, 13% of the global carbon budget (around 5600 million tons CO_2 equivalent) could be due to production and management of petrochemical plastics. Thus, to avoid exceeding the global temperature to 1.5 °C by 2100 restricting global warming, emissions associated with the production and use of plastics should be managed and decreased (Astrup et al. 2009; World Economic Forum 2016; Rhodes 2018; Zheng and Suh 2019). Therefore, based on the problems previously indicated regarding the depletion of fossil resources, climate change, and environmental damage, serious doubts on sustainability of the current petrochemical-originated plastic manufacturing processes have arisen. Nowadays, there is a growing trend of public concern related to the harmful effects of plastics derived from petrochemical sources on the environment. For this reason, systems to develop sustainable and environmentally friendly alternatives to classic plastics have sparked off great interest.

In this way, biodegradable and compostable materials commonly called bio-based plastics (or bioplastics) have been proposed to emphasize their origin from renewable/sustainable biological resources. It should be pointed that the terms bio-based plastics (or bioplastics), degradable plastics, and biodegradable plastics, commonly used as synonyms, are often improperly used. IUPAC only considers bio-based polymers as bioplastics, indicating that this term corresponds to a plastic derived from renewable feedstocks or issued from monomers derived from biomass (Fig. 21.2). However, not all bio-based plastics are biodegradable or even abiotically degraded. Abiotically, degradation of some plastics, either photodegradable or hydrodegradable plastics, will not readily undergo mineralization, leading to the formation of microplastics with the previously mentioned associated health and environmental problems. Biodegradable plastics undergo biological degradation

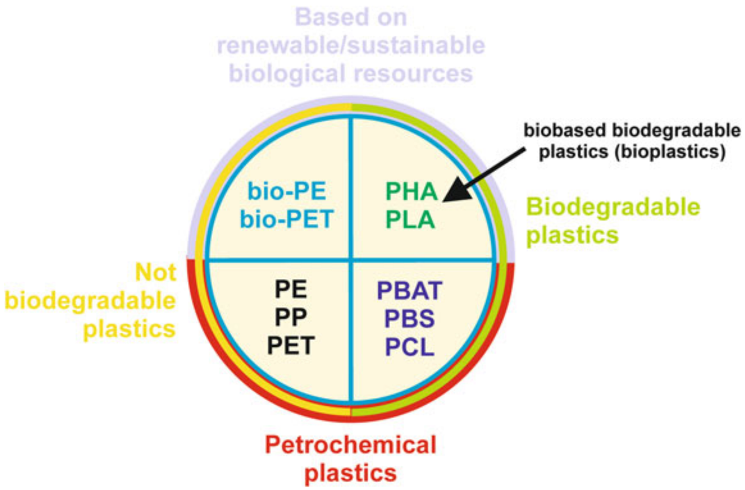

Fig. 21.2 Types of plastics, from conventional petrochemical plastics, both nonbiodegradable (*PE* polyethylene, *PP* polypropylene, *PET* polyethylene terephthalate) to biodegradable (*PBAT* polybutylene adipate-co-terephthalate, *PBS* polybutylene succinate, *PCL* polycaprolactone), to those based on biological resources, biodegradable (*PLA* polylactic acid, *PHA* polyhydroxyalkanoates) and nonbiodegradable (*bio-PE* bio-based polyethylene, *bio-PET* bio-based polyethylene terephthalate)

and full mineralization mainly due to the metabolism of microorganisms. Furthermore, there are bio-based nonbiodegradable plastics that have a similar molecular structure to their petrochemical counterparts (e.g., bio-polyethylene and bio-polyethylene terephthalate), and some petrochemical polymers are considered biodegradable (e.g., polycaprolactone) (Fig. 21.2) (Vert et al. 2012; Lambert and Wagner 2017).

In 2019, the total global production of biodegradable bio-based plastics reached 1.17 million tons and is expected to gain importance in the coming years as production technology improves and market penetration of these products increases, all this being based on a greater social demand for environmentally friendly products (European Bioplastics 2019). Thus, the most promising bio-based and biodegradable alternatives to petrochemical plastics are polylactic acid (PLA) and polyhydroxyalkanoates (PHA) (Fig. 21.3). PLA is a bio-based thermoplastic aliphatic polyester showing properties analogous to polyethylene and polystyrene. This polymer is produced by ring-opening polymerization of lactide or by polycondensation of lactic acid monomers obtained from sugarcane juice or starch fermentation (Drumright et al. 2000; Vink et al. 2004; Auras et al. 2004; Singhvi and Gokhale 2013). Hydrolytically, degradation of this polymer also originates lactic acid.

Chemical synthesis of lactic acid always leads to racemic mixtures of L- and D-isomers of this acid, whereas fermentative production using bacteria, fungi, yeast, cyanobacteria, or algae produces optically pure L(+)- or D(−)-lactic acid or mixtures of both. Almost 90% of lactic acid is produced by biological fermentative processes

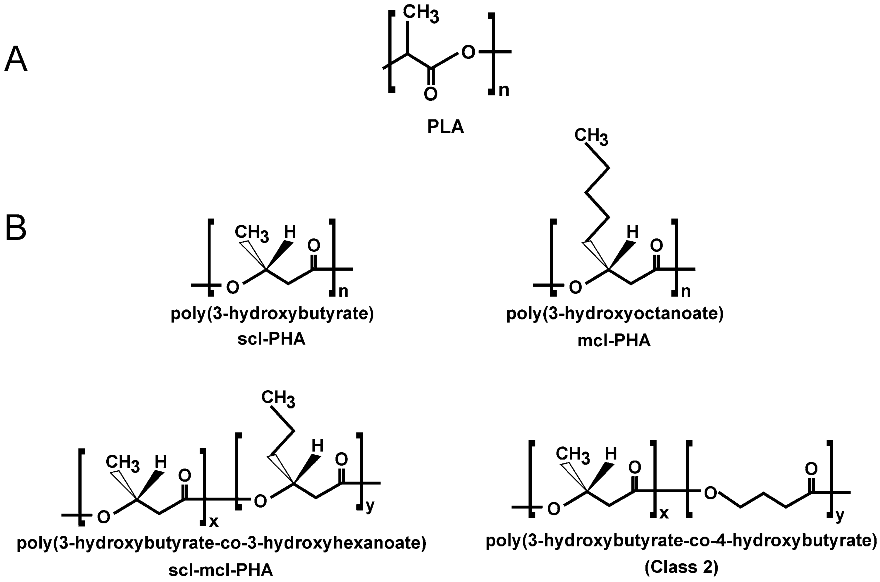

Fig. 21.3 Monomeric structures of **a**, polylactic acid (PLA) and **b**, different polyhydroxyalkanoates (PHAs): poly(3-hydroxybutyrate), a homopolymeric short-chain length PHA (scl-PHA); poly(3-hydroxyoctanoate), a homopolymeric medium-chain length PHA (mcl-PHA); a copolymer composed by poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (scl-mcl-PHA); and a copolymer containing monomers with hydroxylations in C4, poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate)

worldwide, and 10% is chemically produced from petrochemicals (Lunt 1998; Gupta et al. 2007). Considering the amount of L- or D-isomers of lactic acid, polymerization renders pure poly-L-lactic acid, pure poly-D-lactic acid, and poly-D,L-lactic acid (Griffith 2000). Optical purity of lactic acid in PLA is critical as small amounts of enantiomeric impurities can severely alter biodegradability of the polymer (Datta and Henry 2006; Juturu and Wu 2016). Thus, PLA polymers show many excellent properties such as thermoplastics, gas barrier, UV resistant, elastic, rigid, hydrophobic, and biocompatible tissue (Auras et al. 2010; Carosio et al. 2014; Scarfato et al. 2015; Farah et al. 2016).

However, the ever-increasing diversion of starch feedstocks such as maize to PLA production also brings pressures on land use. Thus, for complete substitution of about 360 million tons of petrochemical plastics currently produced by PLA (or bio-based materials synthesized from starch), 11% of cultivable land should be destined for that purpose, leading to a serious conflict between using land for food or plastic (Ragauskas et al. 2006; Rhodes 2019).

21.2 Polyhydroxyalkanoates (PHAs)

Under PHA denomination, a high number of different hydrophobic intracellular polymeric inclusions synthesized by archaea, cyanobacteria, and a wide range of bacteria, both gram positive and gram negative, are grouped (Lu et al. 2009; Han et al. 2010, 2014; Hermann-Krauss et al. 2013; Anjum et al. 2016; Ng and Sudesh 2016; Koller 2019). All of these are optically active linear bio-polyoxoesters that differentiate by monomer composition, (*R*)-hydroxy fatty acids, usually showing the hydroxylation in C3, but not exclusively (Fig. 21.4). Thus, additional variety in PHA stems through the incorporation of 4-, 5-, and 6-hydroxy fatty acids (Kunioka et al. 1989; Valentin et al. 1992, 1994, 1996; Eggink et al. 1995). Hydroxyalkanoic acids are all in the *R* configuration due to the stereospecificity of the polymerizing enzymatic machinery (Anderson and Dawes 1990; Madison and Huisman 1999; Luengo et al. 2003). Ester bonds in the polymer are produced by connecting the carboxylic group of a monomer with the hydroxyl group of a neighboring one.

Summarizing the properties of PHAs, besides bona fide biodegradability, these polymers are generally water insoluble but soluble in few organic solvents, mainly halogenated hydrocarbons. They show a higher density than water, which facilitates biodegradation allowing for their deposition in sediments in aquatic environments, and are relatively resistant to hydrolytic degradation, although they show poor resistance to acids and bases and are also resistant to UV radiation. They offer good resistance to moisture and aromas. However, as an inconvenience, some PHAs

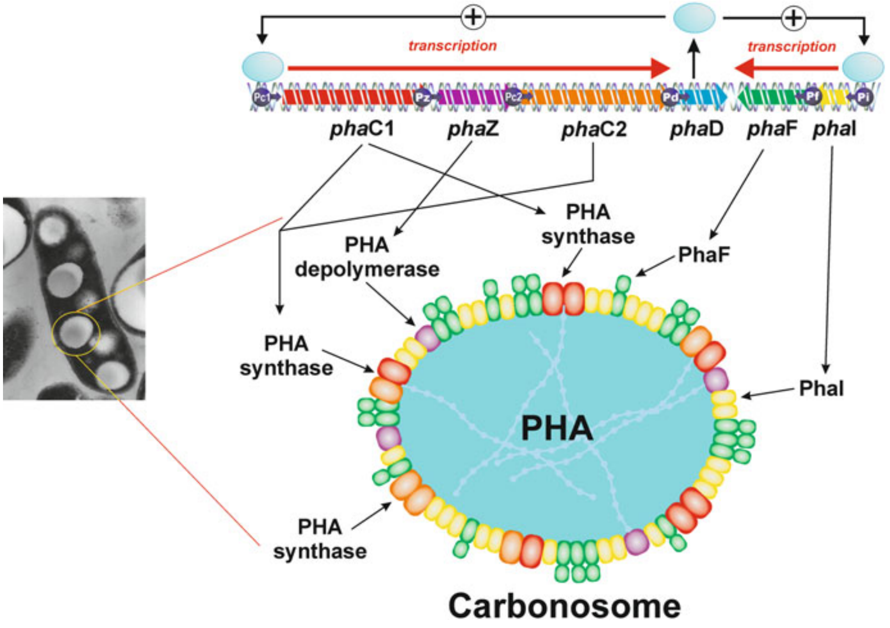

Fig. 21.4 Genetic organization of *pha* cluster and carbonosome in *Pseudomonas putida* U

show a tendency to recrystallize with aging, suffering a loss of flexibility and elongation capacity which can be avoided by blending with other polymers or by using of plasticizers (Bugnicourt et al. 2014).

Classically, PHAs have been classified depending on the number of carbon atoms in the alkyl groups from (*R*)-3-hydroxy fatty acid monomers. Thus, considering the length of the monomer, two different groups could be established, short-chain length PHAs (scl-PHAs), containing monomers of 3–5 carbon atoms, and medium-chain length PHAs (mcl-PHAs), with monomers with 6 carbon atoms or longer (Fig. 21.3) (Madison and Huisman 1999; Aldor and Keasling 2003). Moreover, PHAs could be synthesized by producer organisms as homopolymers, when only one type of monomer is present in the polymer, or as heteropolymers or copolymers, even terpolymers, when different (*R*)-3-hydroxyacyl monomers are found in the same polymer (Fig. 21.3) (Madison and Huisman 1999; Olivera et al. 2001a, b; Luengo et al. 2003). Increasing the possibilities of variation on PHAs, incorporation of monomers on the polymer chain could be controlled through substrate feeding manipulation, allowing the obtaining of random, block, or random-block copolymers (Pederson et al. 2006; Laycock et al. 2013).

Up to now, more than 150 different monomers have been identified as constituents of PHAs in different bacteria, including unsaturated or functionalized fatty acids (Steinbüchel and Valentin 1995; Kim et al. 2007; Olivera et al. 2010). Such structural diversity allows for the obtaining of PHAs showing a high variability in melting, glass transition, and thermodegradation temperatures, with a wide spectrum of mechanical properties including a very flexible Young's modulus, elongation at break, and tensile strength (Chen 2010; Volova et al. 2013). Composition of PHA polymers depends on the carbon compound supplied as carbon source to support growth and as source of monomeric precursors, bacterial strain used, and metabolic plasticity of the strain.

Highlighting this great variability of properties in function of composition of these polymers, the case of poly(3-hydroxybutyrate) (PHB) could be consider as example. PHB, the most well-known scl-PHA member, shows good thermoplastic properties, although it has poor mechanical properties, as it is a stiff and brittle material difficult to be processed due to its crystalline nature (Sudesh et al. 2000). Co-metabolism of valerate in some PHB-producing bacteria allows for the incorporation of 3-hydroxyvalerate monomers inside PHB, resulting in the production of the copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), a material which becomes tougher, more flexible, and broader to thermal processing when molar fraction of 3-hydroxyvalerate in the copolymer increases (Lee 1996a). Scl-PHAs are mostly used to produce disposable items and food packaging materials. On the other hand, mcl-PHAs are characterized such as elastomers, elastic or glue-like materials with a low degree of crystallinity, and low melting temperatures (Sudesh et al. 2000; van der Walle et al. 2001).

To sum up, plasticity of bacterial metabolism makes it possible to control monomeric composition in PHAs by choosing the suitable carbon sources, producers (both wild-type and genetically engineered strains), and fermentation conditions. Therefore, tailoring of physical properties of the polymer, a fact that is very difficult or even impossible by chemical synthesis, became possible. Moreover, to achieve another improved level on polymer characteristics, PHAs can also be blended or grafted with other polymers (Yu et al. 2006). Furthermore, side chains of the PHAs can be chemically modified introducing a desired functional group into a natural PHA, modifying material properties of the polymers (Hartmann et al. 2006). Thus, due to all these possible variations, as a function of specific monomeric composition and affecting their physicochemical and mechanical properties, design of specific bio-based technical-grade polymers is fully permitted, ranging from tough and rigid to flexible and elastomeric, and offering opportunities for replacing petroleum-derived polymers, and setting the stage for the synthesis of new materials tailored for specific applications, such as drug delivery nanodevices, tissue engineering matrices, surgical sutures, and implants (Abraham et al. 2001; Olivera et al. 2001b; Chen and Wu 2005; Gao et al. 2011; Luef et al. 2015; Nakajima et al. 2017).

From a physiological point of view, polyhydroxyalkanoates (PHAs) are bacterial carbon storage lipidic compounds accumulated in the cytoplasm of a wide variety of bacteria as discrete granules (carbonosomes) when these producer organisms grow on an oversupply with carbon source (Anderson and Dawes 1990; Jendrossek 2009; Jendrossek and Pfeiffer 2014). The number and size of PHA granules inside cells, monomeric composition of accumulated PHA, and macromolecular structure and characteristics vary depending on the bacterial strain accumulating these granules, as well as the inception of polymerizable monomers (Anderson and Dawes 1990; Madison and Huisman 1999; Luengo et al. 2003; Olivera et al. 2010).

Although the accumulation of these storage granules has been classically associated to bacterial growth under limiting conditions, such as nitrogen, phosphorus, or oxygen starvation, always with an excess of an available carbon source in the environment (Schlegel et al. 1961; Senior et al. 1972; Schembri et al. 1995a; Shi et al. 2007; Albuquerque et al. 2010), production of PHAs has been also reported in some microorganisms under non-stressing growth by impairment of nutrient availability (some strains belonging to *Pseudomonas putida, Sphaerotilus natans, Bacillus mycoides, Azotobacter vinelandii, Alcaligenes latus,* and others) (Page and Knosp 1989; Hänggi 1990; Huisman et al. 1992; Takeda et al. 1995; Wang and Lee 1997; García et al. 1999; Borah et al. 2002). This discrepancy between the ability to accumulate PHAs under limited nutrient conditions and unrestricted growth has consequently an important implication at industrial production of these polymers: PHA production at last exponential growth phase or even at stationary phase versus production at exponential growth phase. These different behaviors clearly show distinctive traits in the regulatory mechanisms controlling PHA production between different bacterial strains, which need to be more deeply explored.

It has been classically recognized that the role of PHAs is to act as a depository for carbon and reducing equivalents (Senior and Dawes 1973; Madison and Huisman 1999). However, it is now known that PHAs are more than an intracellular carbon storage compound mobilizable as fatty acids under famine conditions since it has also been shown that PHA accumulation enhances the survival of certain bacteria under environmental stress conditions, including high or low temperatures, in so much that PHAs provide enhancement of robustness and survival rate and protect bacteria from damage by extracellular ice during freezing, osmotic shock-stabilizing cytoplasmic membranes, oxidative pressure, or exposure to UV irradiation (Tal and Okon 1985; Kadouri et al. 2005; Zhao et al. 2007; Wang et al. 2009; Obruca et al. 2010). Moreover, accumulation of PHAs by bacteria dependent on photosynthetic carbon fixation from microbial mats helps to maintain anoxic photosynthesis and sulfur cycle, participate in the spark of sporulation in some bacilli, and allow for the maintaining of nitrogen fixation in the dark by some diazotrophs (Slepecky and Law 1961; Bergersen et al. 1991; Urmeneta et al. 1995; Rothermich et al. 2000).

Intracellular PHA granules are typically 100–500 nm in diameter (Sudesh et al. 2000). These PHA inclusions consist of hydrophobic polyester core surrounded by an amphiphilic layer acting as a surfactant-like barrier that avoids the spread of hydrophobic polymer in aqueous cytoplasmic environment (Fig. 21.4). Until recently, the existence of a phospholipidic monolayer surrounding the polyester, into which different proteins were embedded, had been accepted, mainly based on the identification of phosphatidic acid and other phospholipids in purified poly 3-hydroxybutyrate granules from *Bacillus megaterium* by Griebel and from *Cupriavidus necator* (formerly *Ralstonia eutropha*) by Horowitz and Sanders (Griebel et al. 1968; Horowitz and Sanders 1994). However, it has been shown that the phospholipids detected in the isolated granules were artifacts throughout the isolation procedure. Electron microscopy micrographs of granules from *Rhodospirillum rubrum* and *C. necator* showed the existence of a monolayer of 3–4 nm thickness surrounding the inclusions that initially was interpreted as a phospholipidic layer in which different proteins could be incorporated (Boatman 1964; Mayer and Hoppert 1997). In fact, it has been shown that the layer covering the granules has a proteinaceous nature being made up of the so-called PHA granule-associated proteins, and size and structure of the amphiphilic layer surrounding the granule observed by electron microscopy do not enter in conflict with a structure formed exclusively by proteins (Pötter and Steinbüchel 2006; Beeby et al. 2012; Bresan et al. 2016). These layers include enzymes involved in the synthesis of the polymer and PHA synthases (PhaC) and depolymerization of monomers from polymer, PHA depolymerases (PhaZ), structural proteins so-called phasins (PhaP, PhaF, PhaI), and, in some cases, regulatory proteins (PhaR) (de Koning and Lemstra 1992; Wieczorek et al. 1995; Foster et al. 1996; Stuart et al. 1996; Sudesh et al. 2000; York et al. 2002; Rehm 2003; Jendrossek 2007; Beeby et al. 2012; Mezzina and Pettinari 2016; Bresan et al. 2016; Maestro and Sanz 2017; Long et al. 2018). Remarkably, association of other proteins involved in synthesis or mobilization of monomer precursors in the layer has also been suggested (Uchino et al. 2007; Ruth et al. 2008; Ren et al. 2009b).

Although PHA granule structure and metabolic functions for PHA polymerization/depolymerization and granule conformation seem to be maintained in all the organisms able to produce them, both genes and enzymes involved are different between groups of microorganisms. Thus, apart from the genetic organization maintained between groups, PHA synthases, the enzymes in charge of (*R*)-3-hydroxyacyl-CoA (3HA-CoA) thioester monomers' incorporation into the polymer, define the synthesized PHA (scl- or mcl-PHA) and reflect the genetic organization of

PHA synthesis and mobilization in the different groups. Thus, PHA synthases, all of them belonging to α−/β-hydrolase family of proteins, are grouped into four classes based on constituent subunits, amino acid sequence, substrate specificity, and the preference in forming scl- or mcl-PHAs (Rehm 2007): class I, class III, and class IV produce mainly scl-PHAs, with class I and III synthases generally having additional low affinities for ≥6 carbon mcl-3HA-CoA precursors, although this depends on species (Sudesh et al. 2000). However, *Aeromonas caviae* and *R. rubrum* having a class I synthase, and *Thiocapsa pfennigii,* which presents a class III synthase, are able to synthesize mcl-PHAs containing 3-hydroxyhexanoate or 3-hydroxyoctanoate when provided with carbon sources of these lengths (Brandl et al. 1989; Fukui and Doi 1997; Liebergesell et al. 2000). *C. necator, Aeromonas* spp., or the core genome of *Comamonas* present class I PHA synthases (Peoples and Sinskey 1989a; Fukui and Doi 1997; Wu et al. 2018). On the other hand, class II enzymes, the canonical class present in pseudomonads and in *Halomonas* spp., utilize medium-chain length 3-HA-CoA monomers to synthesize mcl-PHAs (Rehm 2003; Ilham et al. 2014).

Class I and class II synthases only consist of one type of subunit PhaC (61–73 kDa) for activity (Qi and Rehm 2001), while classes III and IV require an additional subunit, PhaE or PhaR, respectively. Thus, class III PHA synthases, present in, for example, *Allochromatium vinosum* (formerly *Chromatium vinosum)* and *Haloarcula marismortui*, are composed of a catalytic subunit (PhaC, 40–53 kDa) and a second subunit (PhaE, 20 or 40 kDa), forming a PhaEC complex, in which the PhaE subunit is necessary for PHA polymerization; class III PhaC are structured as tetramers (Han et al. 2007; Zhang et al. 2015). Similarly, class IV PHA synthases, appearing in *Bacillus* spp. as model organisms, are composed of a PhaC catalytic subunit (40 kDa) associated to PhaR (20 kDa) forming the PhaRC complex (Tariq et al. 2015; Tsuge et al. 2015).

A close cooperation between PhaC and phasins has been shown in *C. necator* H16 suggesting that these proteins are key functions in the structuration of the carbonosome and in the interaction of PHA granules, DNA, and the enzymes involved in biosynthesis and depolymerization of PHA and determining the granule localization within the cell (Pfeiffer et al. 2011; Jendrossek and Pfeiffer 2014; Obeso et al. 2015a). Phasins, the most abundant proteins in the carbonosome (Mayer et al. 1996), have been observed associated to the carbonosomes of all PHA-producing bacteria, for example, *C. necator* (which has seven identified phasins PhaP1-PhaP7) (Wieczorek et al. 1995; Neumann et al. 2008; Pfeiffer and Jendrossek 2012), *A. vinosum* (Liebergesell 1992), *R. ruber* (Pieper-Fürst et al. 1994, 1995), *R. rubrum* (ApdA protein) (Handrick et al. 2004), *Paracoccus denitrificans* (Maehara et al. 1999), *P. putida* (Prieto et al. 1999; Sandoval et al. 2007; Galán et al. 2011; Obeso et al. 2015a), *Methylobacterium* sp. (GA20 and GA11 proteins) (Föllner et al. 1997), *Magnetospirillum gryphiswaldense* (Mms16 protein) (Schultheiss et al. 2005), *Acinetobacter* sp. (Schembri et al. 1995b), *Bacillus megaterium* (McCool and Cannon 1999, 2001), and even in *Haloferax mediterranei*, an *Archaea* (Cai et al. 2012). Thus, phasins are synthesized simultaneously to PHA synthesis to form the coating of the hydrophobic polymer surface and therefore protecting the host cell and participating in the process of distribution of granules during cell division. Moreover, it has been suggested that binding of phasins prevents unspecific attachment of proteins to carbonosome surface (Neumann et al. 2008).

Another interesting point to consider is the state of the hydrophobic polyester core inside the carbonosome. Once extracted from cells or after cell lysis at ambient conditions and depending on the specific molecular structure of PHA, structure of the pure polymer generally shows a high degree of crystallinity and the polyester chains tend to adopt an ordered helical conformation (Merrick and Doudoroff 1964; Cornibert et al. 1972; de Koning and Lemstra 1992). However, PHA core inside the carbonosome maintains a hydrated metastable amorphous state, with mechanical properties resembling supercooled liquids, allowing for some mobility and flexibility of the polymer chains. Thus, this amorphous state is due to the plasticizing action of water. Reinforcing that, presence of residual water inside native PHA granules, at about 5–10% by mass, has been shown, which may be interacting with the ester groups either from the polymer hydrogen bond or from dipole-dipole interactions (Barnard and Sanders 1989; Bonthrone et al. 1992; Lauzier et al. 1992).

Alternative mechanisms of protection of PHA against crystallization solely dependent of crystallization kinetics has been proposed, in which amorphous PHA is restricted by the submicron volume of the granule where the frequency of nucleation needed for crystallization is extremely low (Bonthrone et al. 1992; de Koning and Lemstra 1992; Obruca et al. 2020). Recently, it has been proposed that the hydrated metastable state may be further stabilized by Ca^{2+}-coordinated organization, suggesting the localization of this ion in the carboxyl end-group region from polymer chain. Moreover, these terminal carboxyl groups are not exposed to the carbonosome surface but buried inside mature granules allowing for both structuration of the granule and storing Ca^{2+} inside the granule for regulation of its cytosolic level (Tian et al. 2019). The maintaining of metastable amorphous state of polymer inside the carbonosome could probably be due to a synergistic interaction of these factors together with an effect promoted by the surface proteins maintaining granule structure. Physiologically, proposed amorphous in-cell structure of polymers inside PHA granules is completely mandatory since intracellular enzymes in charge of mobilization (hydrolysis) and catabolism of the stored polymer (PHA depolymerases) are not able to accomplish their function on crystalline PHA (Jendrossek 2007, 2009; Sedlacek et al. 2019).

Moreover, it has been recently proposed that after exposure of PHA accumulating bacterial cells to some stressor agents, function of the granule-associated proteins that conform the surface layer of the carbonosomes could be lost, inducing coalescence of the granules, increasing the effective volume of the polymer, and after dehydration of the cells, residual water is excluded from the granule triggering off crystallization of the polymer. Thus, this proposal suggests that although bacterial resistance to some stressing injury could be due to the intimal interconnection of PHA metabolism with central pathways of the cellular metabolism providing metabolic fuels or allowing for the maintenance of a redox state, physical nature of internal PHA itself could be another mechanism for survival to different stressors (de Koning and Lemstra 1992; Sanders 1993; Sedlacek et al. 2019).

21.3 Medium-Chain Length Polyhydroxyalkanoates

As previously indicated, medium-chain length PHAs (mcl-PHA) have elastomeric and thermal properties which generally make them superior bioplastics for rubber and viscoelastic applications when compared to scl-PHA (Sudesh et al. 2000). While numerous organisms have been studied for their natural or recombinant production of scl-PHAs, i.e., poly(3-hydroxybutyrate) (PHB), poly(3-hydroxyvalerate), and/or copolymers of them, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), fewer organisms are natural producers of mcl-PHAs, being the species belonging to genus *Pseudomonas* the more extensively studied. Thus, most of the research on the proteins involved into structuration and biosynthesis of PHAs and the production of monomers for the synthesis of mcl-PHA, as well as production and characteristics of natural and unusual mcl-polymers, has been made using *P. putida* strains GPo1 (formerly *P. oleovorans*) (de Smet et al. 1983; Huisman et al. 1991), *P. putida* KT2440 (and its rifampicin-resistant mutant KT2442) (Rehm et al. 1998; de Eugenio et al. 2007), *P. putida* U (García et al. 1999; Abraham et al. 2001; Luengo et al. 2003; Sandoval et al. 2005; Obeso et al. 2015a, b), and *Pseudomonas* sp. 61–3 (Matsumoto et al. 2002; Hokamura et al. 2015).

Thus, synthesis and polymerization of (*R*)-3HA-CoA monomers which contain from between six (3-hydroxyhexanoyl-CoA) and 14 carbons (3-hydroxytetradecanoyl-CoA) allow for the accumulation of mcl-PHAs in pseudomonads. With regard to the length and composition of monomeric side chains, including unusual monomers structurally related to natural 3HA-CoAs due to the wide versatility of pseudomonads, such as alkanoates containing exotic substituents, and the proportion of different monomers in the polymerized material, mcl-PHAs produced by *Pseudomonas* spp. are highly variable. Interestingly, a few strains of *Pseudomonas* are able to synthesize a mixture of scl- and mcl-PHAs, such as *Pseudomonas* sp. 61–3, *Pseudomonas* sp. USM 4–55, *Pseudomonas* sp. LDC-5, *P. oleovorans* B-778, *P. stutzeri* 1317, and *P. pseudoalcaligenes* YS1 and CECT5344, since these strains have two separate biosynthetic pathways with different PHA synthases, one for scl-PHAs and the other for mcl-PHAs (Matsusaki et al. 1998; Ashby et al. 2002; Hang et al. 2002; Chen et al. 2004; Sujatha et al. 2007; Tan et al. 2010; Manso Cobos et al. 2015).

Carbonosomes of pseudomonads mcl-PHAs range from one to approximately ten inclusions per cell and are generally arranged around the center of the longitudinal axis of the cell (Beeby et al. 2012). These carbonosomes also have a proteinaceous surface layer lacking phospholipidic compounds (Bresan et al. 2016). In these bacteria, proteins involved into mcl-PHA biosynthesis are encoded by the *pha* cluster (Fig. 21.4). This cluster is organized into two operonic structures, *phaC1ZC2D*, encoding two different PHA synthases (PhaC1 and PhaC2), an intracellular PHA depolymerase (PhaZ), and PhaD, a regulatory protein, and *phaIF*, encoding two phasins-like proteins, PhaI and PhaF, both belonging to the Pfam PF05597 family of phasin proteins (Huisman et al. 1991; Prieto et al. 1999; García et al. 1999; de Eugenio et al. 2007, 2010a; Galán et al. 2011). PhaD, although important for the regulation of mcl-PHA formation (see below), is apparently the only one of these proteins that has not been found bounded to carbonosome in vivo (Klinke et al. 2000; de Eugenio et al. 2010b; Tarazona et al. 2020). Moreover, acyl-coenzyme A synthetase 1 (Acs1), an enzyme involved in the mobilization of the monomers from granule, was identified in *P. putida* to be attached to pseudomonads PHA granules in vivo via co-localization of translational fusions with GFP. Remarkably, other proteins involved in fatty acid metabolism such as FabG, FadE, FadL, and a putative acyl-CoA dehydrogenase have been identified recently as attached to carbonosome. These findings suggest that enzymes involved in PHA precursor synthesis and mobilization can also specifically bind to PHA granules (Ruth et al. 2008; Ren et al. 2009b; Tarazona et al. 2020).

Although it has been classically admitted that the genes for PHA synthesis in *Pseudomonas* spp. constitute two divergent operons (*phaC1ZC2D* and *phaIF*), in *P. putida* U, functional promoters for each individual gene have been identified (Fig. 21.4) (Sandoval et al. 2007). Moreover, *P. putida* KT2442 promoter sequences have been identified upstream of each of the genes, except for *phaD* (Galán et al. 2011). In this way, in *P. corrugata* strains CFBP and 388, no polycistronic transcript of *phaC1ZC2D* was expressed under any culture condition for PHA production assayed, indicating that *phaC1* and *phaC2* in these strains are not cotranscribed (Conte et al. 2006). Moreover, mRNA analyses in *P. putida* GPo1 suggested that *phaC1*, *phaZ*, *phaC2,* and *phaD* do not form part of the same transcription unit. However, in this strain and in *P. aeruginosa*, *phaC1* and *phaC1Z* transcriptional units have been reported. Thus, in these strains, the transcription should partially stop at the end of the *phaC1* gene and partially continue to the end of *phaZ* (Timm and Steinbüchel 1992; Prieto et al. 1999).

According to the expression of *phaC1* as a monocistronic mRNA, a transcriptional terminator in the intergenic sequence between *phaC1* and *phaZ* has been described in the strain *P. corrugata* 388, affecting the expression of *phaZ* and *phaC2* (Solaiman et al. 2008). Analogous transcriptional terminator between *phaC1* and *phaZ* has been identified in several other *Pseudomonas* strains (e.g., *Pseudomonas* sp. 61–3 and *P. pseudoalcaligenes* HBQ06), although it is absent in other strains (e.g., *P. putida* KT2440 and *Pseudomonas* sp. LDC-25) (Ciesielski et al. 2011).

On the other hand, in *P. putida* GPo1, a repetitive extragenic palindromic sequence which could act as a Rho-independent transcriptional terminator has been identified downstream of the *phaZ* gene (Huisman et al. 1991; Aranda-Olmedo et al. 2002). Interestingly, this putative transcriptional terminator sequence seems to be absent in *P. putida* KT2442, supporting the transcription of a polycistronic *phaC1ZC2D* mRNA expressed under the government of the promoter sequence upstream *phaC1*. Additionally, the lack of promoter immediately upstream of *phaD* suggested that the expression of this gene is controlled by the *phaC1* upstream promoter (de Eugenio et al. 2010b).

Nevertheless, in *P. putida* GPo1 and *P. aeruginosa*, two promoters have been reported upstream of the *phaC1* gene, being the closest to *phaC1* ribosome binding site a promoter showing dependence by RpoN (RNA polymerase σ^{54} factor) although the other promoter seems to present RpoD (σ^{70})-dependent binding sites (Huisman et al. 1991; Timm and Steinbüchel 1992). Downstream of *phaD* and between this gene and *phaF*, in *P. putida* U, GPo1, and KT2442, another putative transcriptional terminator has been proposed in base to the existence of an extragenic palindromic sequence. Taking into account that the intergenic sequence in these strains does not maintain a high level of similarity, the presence in all of them of this kind of structure highlights its functionality, probably acting from a topological mechanism and favoring relaxation of supercoiled DNA in this region affecting at transcription of surrounding genes.

When the transcription of *phaF* gene was analyzed in *P. putida* PGo1, this gene was seen as transcribed in two different mRNAs, one containing exclusively *phaF* and the other containing both *phaI* and *phaF* genes, suggesting the possible presence of two promoters, one located upstream of the *phaI* gene, allowing for the transcription of a bicistronic mRNA containing the information for PhaI and PhaF, and the other promoter located upstream of *phaF*, driving the transcription of a monocistronic mRNA (Prieto et al. 1999). Similar results have also been observed for *P. putida* KT2440 and *P. aeruginosa* (Hoffmann and Rehm 2004, 2005).

21.3.1 PHA Synthases

PhaC synthases remain attached to the carbonosome being a key component of the proteinaceous layer surrounding the polymer. These enzymes catalyze the conversion of (R)-3HA-CoAs comprising 6 to 14 carbon atoms into mlc-PHAs with the concomitant release of CoA (Rehm and Steinbüchel 1999; Amara and Rehm 2003). Classically, it has been stated that PHA synthases from pseudomonads are homodimers composed of PhaC1 or PhaC2 monomers (Zou et al. 2017). However, experimental evidences suggest that in *P. putida* U, heterodimers composed by PhaC1 and PhaC2 are also formed (Obeso et al. 2015b). Thus, when PhaC1 and PhaC2 were independently expressed in a mutant derivative of *P. putida* U lacking the whole *pha* cluster (*P. putida* Δ*pha*), both proteins were able to polymerize 3HA-CoAs (Fig. 21.5). However, simultaneous expression of either proteins or mutant inactive PhaC1 versions together with functional PhaC2, in this genetic background, showed that in this strain, a functionally active PhaC1 is an absolute requirement for a functionally active PhaC2, suggesting the interaction of both proteins. This interaction was also supported through in silico docking simulations using bioinformatic models of the tertiary structure of these proteins (Obeso et al. 2015b).

When *phaC1* and *phaC2* from *P. aeruginosa*, the genes encoding the two PhaC synthases, were expressed separately in recombinant *E. coli*, the highest yield of PHA was obtained when decanoate or dodecanoate was used as a source of precursors and a PHA containing 3-hydroxydecanoate as the mayor monomer

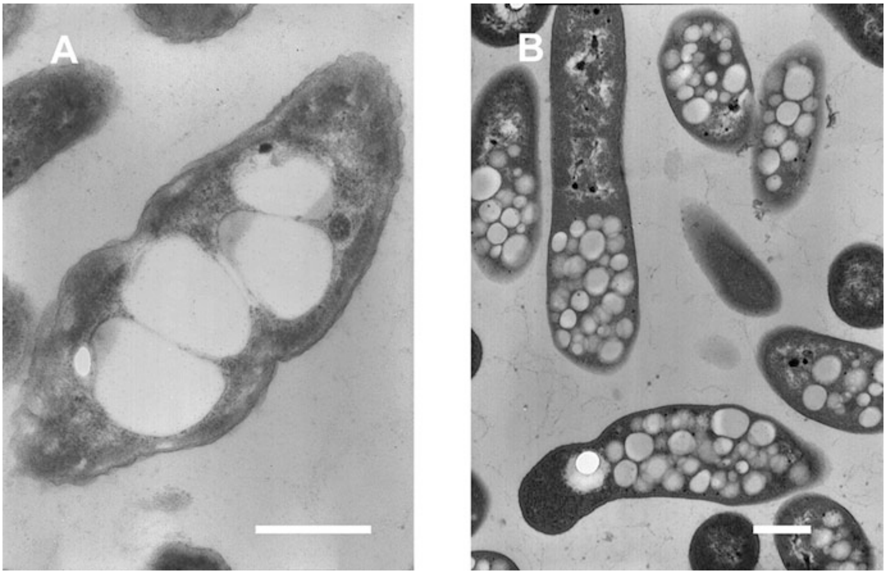

Fig. 21.5 Effect of phasins in structure and distribution of the carbonosomes in *P. putida* U. (**a**) Transmission electron microscopy picture of wild-type strain; (**b**) transmission electron microscopy of *P. putida* Δ*pha* pMC*phaC1*, a mutant of the wild-type strain in which whole *pha* cluster has been deleted, and PHA synthase (*phaC1*) is provided using *trans*-expression from a plasmid (bar indicates 500 nm)

constituent was found. In the recombinant strains, both enzymes showed similar substrate specificities (Langenbach et al. 1997; Qi et al. 1997). Analysis of the PhaC1 and PhaC2 synthases from *P. mendocina* NK-01 revealed that both enzymes have also similar substrate specificities although PhaC1 displays a higher catalytic rate and produces lower molecular weight PHAs than PhaC2 (Guo et al. 2013). However, studies published using other *Pseudomonas* strains have shown that PhaC1 and PhaC2 from the same strain showed obvious differences in substrate specificity (Hein et al. 2002; Chen et al. 2004).

Moreover, studies performed with *phaC1* and *phaC2* from *P. mendocina* strain DSM50017 showed that PhaC1 is the major enzyme for PHA synthesis in this strain, whereas PhaC2 only contributes, in a lesser extent, to PHA synthesis and only when this strain is cultivated on carbon sources not related to fatty acids (Hein et al. 2002). Studies conducted using *P. putida* Δ*pha* as host for expression of *phaC1* or *phaC2* from the wild-type *P. putida* U strain showed that in this strain, both polymerases show quite different substrate specificity. Thus, whereas PhaC1 is able to polymerize several aliphatic and aromatic 3HA-CoAs, with 3-hydroxydecanoyl-CoA being the best substrate for polymerization, PhaC2 is able to polymerize an aromatic 3HA-CoA derivative (3-hydroxy-5-phenylvaleryl-CoA) and certain aliphatic ones, being 3-hydroxyhexanoyl-CoA recognized as the best substrate (Arias et al. 2008).

The PhaC subunit has a lipase boxlike sequence, G-X-C-X-G, where X is an arbitrary amino acid. The Cys in this lipase boxlike sequence (Cys296 in PhaC1 and PhaC2 from *P. putida* U) is the active center of PHA synthases. A bioinformatic model based on homology on the folding of PhaC1 and PhaC2 from *P. putida* U showed that both proteins acquired an α/β hydrolase fold covered by a "lid" (residues 330–425) burying the predicted catalytic triad (Cys296—Asp451—His479 for PhaC1, Cys296—Asp452—His480 for PhaC2). Cys296 could act as a nucleophile, attacking the activated carboxyl group of 3HA-CoAs, displacing the CoA, and giving the acyl-enzyme intermediate which should react with the free hydroxyl group of another 3HA-CoA. Asp452/453 acts as a general base catalyst to accelerate deprotonation of the 3-hydroxyl group of polymers in the step involving incorporation of 3HA-CoAs. Moreover, Ser325/326 located close to the catalytic His479/480 stabilizes the catalytic Cys through hydrogen bonding. The lid comprising of residues 330–425 has been suggested to be involved in interfacial activation of these proteins (Arias et al. 2008; Obeso et al. 2015b). Moreover, these models are reinforced by the results obtained in the study of crystalized PHA synthases from *C. necator* H16 and *Chromobacterium* sp. USM2 (Wittenborn et al. 2016; Kim et al. 2017a, b; Chek et al. 2017, 2020).

Much interest is centered on engineering the single subunit of class II PHA synthases to improve their activities, substrate specificities, product polymer characteristics, and stabilities (Nomura and Taguchi 2007; Zou et al. 2017).

21.3.2 PHA Depolymerase

PhaZ is a PHA depolymerase which allows for the hydrolysis of accumulated biopolymers so that its products can then feed into central catabolism through β-oxidation (Fig. 21.5) (Huisman et al. 1991; Foster et al. 1996; García et al. 1999; Sandoval et al. 2005; de Eugenio et al. 2007). In *P. putida* CA-3, under carbon-limiting conditions, only transcription of the *phaZ* depolymerase gene was observed concurrent with the absence of detectable PHAs, demonstrating their function as the enzyme in charge to mobilize PHA (O'Leary et al. 2005). Surprisingly, PhaZ depolymerase is produced both under PHA production and mobilization growth conditions, being permanently associated to the carbonosomes, and during polymerization of PHA, PhaZ releases monomers from this polymer (Ren et al. 2009a, 2010; de Eugenio et al. 2010a).

PhaZ belongs to family V of bacterial lipolytic enzymes containing a potential lipase box (Arpigny and Jaeger 1999). Based on a bioinformatic model of PhaZ from *P. putida* KT2442, this protein consists of a core domain with α/β-hydrolase fold and a lid comprising of residues 133–192. Three residues, Ser102, Asp221, and His248, constitute a catalytic triad, in which Ser102 is able to nucleophilically attack the carbon atom in the ester bond, linking the monomers in PHA, due to the location of the triad near the hinge connecting the lid and the core domain (de Eugenio et al. 2007, 2008).

Removal of *phaZ* has been proposed as a strategy to increase PHA production. However, impact of the knockout of this gene on PHA yield in *Pseudomonas* is controversial. Some studies have reported that loss of the depolymerizing enzyme triggers an increase in PHA production in *P. putida* U, *P. putida* KT2442, and *P. putida* AG2228 (García et al. 1999; Cai et al. 2009; Arias et al. 2013; Salvachúa et al. 2020) although little or no impact has been described in PHA accumulation in *P. putida* KT2440 and *P. resinovorans* (Vo et al. 2015; Zhou et al. 2020).

21.3.3 PhaD Regulatory Protein

PhaD is a TetR family transcriptional regulator in *P. putida* GPo1 (Klinke et al. 2000), *P. putida* U (Sandoval et al. 2007), and *P. putida* KT2440 (de Eugenio et al. 2010b). In these strains, this transcriptional regulator positively affects PHAs synthesis. Thus, it has been described that *phaD* knockout reduces the amount of polymer accumulated by *P. putida* GPo1, increasing the number of granules inside the cells although decreasing their size drastically. These effects have been related to a decrease in phasin levels, PhaI and PhaF (Klinke et al. 2000). A similar effect has been observed in *P. putida* U (Sandoval et al. 2007).

As previously indicated, a promoter upstream of *phaD* gene has been described in *P. putida* U, which means that in these bacteria, this gene could be transcribed as a monocistronic mRNA although coexpression of this gene as part of polycistronic unit from promoters upstream of *phaC1, phaZ,* or *phaC2* should not be discharged (Fig. 21.4) (Sandoval et al. 2007). However, in *P. putida* KT2442, the *phaD* gene does not have a specific promoter driving its sole transcription, and it is mainly transcribed as part of the *phaC1ZC2D* polycistronic mRNA. Although theoretically this gene could be also expressed from the promoters upstream *phaZ* and *phaC2*, avoidance of the *phaC1ZC2D* transcriptional unit through disruption of the *phaC1* gene demonstrates that *phaD* gene is transcribed mainly from promoter upstream *phaC1* (de Eugenio et al. 2010b). Furthermore, induction of PHA accumulation by PhaD is performed through the binding of this protein to a 25–29 bp operator region of the promoter's upstream *phaC1* and *phaI* activating the expression of the *pha* genes (Fig. 21.4). Interaction of PhaD with operator sequences to develop its transcriptional activation function could be triggered off by the binding of an inducer molecule, probably a CoA-derivative intermediate of fatty acid metabolism (de Eugenio et al. 2010b).

However, in a mutant strain defective of *phaD* derived from *P. putida* KT2442, a basal transcription of *phaC1* and *phaZ* mRNAs was still detectable, suggesting that additional factors might control the expression of the *pha* cluster (de Eugenio et al. 2010b). In this sense, involvement of PhaF phasin in regulation has been pointed out as a player in a possible synergistic mechanism cooperating with PhaD in these regulatory mechanisms (see later). Moreover, as will be discussed later in this chapter, participation of some global transcriptional factors has been demonstrated to be involved in the regulation of *pha* gene expression in different pseudomonads.

21.3.4 Phasins

In mcl-PHA granules of pseudomonads, phasins PhaF and PhaI have been identified as the major constituents of peripheral layer surrounding carbonosome (Fig. 21.4) (Prieto et al. 1999; Sandoval et al. 2007; de Eugenio et al. 2010b). Moreover, these proteins are produced simultaneously to PHA synthesis and play important roles in PHA metabolism (Fu et al. 2015; Beckers et al. 2016; Możejko-Ciesielska et al. 2018; Możejko-Ciesielska and Serafim 2019). The importance of phasins in PHA accumulation has been evidenced in *P. putida* U and *P. putida* KT2442. In a mutant of *P. putida* U in which *phaD* and *phaIF* genes have been deleted, a 75% decrease in the synthesis of PHAs (when using octanoate as carbon source) was observed. It must be considered that the wild-type strain is able to polymerize aliphatic alkanoates (fatty acid derivatives), as well as aromatic monomers synthesized through β-oxidation of ω-phenylalkanoates used as the source of precursors (Abraham et al. 2001; Luengo et al. 2001, 2003; Olivera et al. 2001a). However, in the *phaDphaIF* defective mutant, accumulation of aromatic PHAs was avoided. Moreover, in a derivative of *P. putida* U in which the whole cluster *pha* has been deleted (*P. putida* U Δ*pha*), unable to synthetize PHAs, expression in *trans* of *phaC1* without phasins was enough to produce carbonosomes when using aliphatic precursors although the amount of PHA accumulated was drastically lower than in the wild-type strain, and polymer appeared as a great number of small granules with an abnormal distribution of these inside the cell (Fig. 21.5).

However, no PHA production was observed when phenylalkanoates were used as source of polymer. Moreover, coexpression in this genetic context of *phaC1* and *phaD* or *phaC1* and *phaI* failed to modify either the number or size of the granules, whereas when *phaC1* and *phaF* were expressed together, the recombinant bacteria restored the number, distribution, and size of carbonosomes resembling the wild-type strain. Surprisingly, when *phaC1* was coexpressed with a construction expressing *phaIF* genes from their natural promoters, the recombinant strain accumulated very small granules of PHA, suggesting the need of PhaD regulatory protein for the correct transcription of phasins; when PhaD was coexpressed in this recombinant strain, a phenotype similar to wild-type strain was reached. These results indicate the importance of phasins for correct structuration of the carbonosomes in *P. putida* U and the function of PhaD as transcriptional regulator of PHA synthesis in these bacteria (Sandoval et al. 2007). Reinforcing this, deletion of *phaF* in *P. putida* KT2442 resulted in a falling to 7% of cell dry weight in PHA production, a reduction in the number and size of PHA granules, as well as aberrant location inside the cells, also affecting the segregation of these granules during cell division (Prieto et al. 1999; Galán et al. 2011; Dinjaski and Prieto 2013).

Bioinformatics modelling of PhaF suggests that this protein is a tetramer made up of elongated protomers. In each protomer, three different domains can be differentiated: (1) a long, amphipathic N-terminal helix; (2) a short leucine zipper, a proposed signature motif for protein-protein interaction and homo- and hetero-oligomerization (Tarazona et al. 2020); and (3) a superhelical C-terminal domain. This C-terminal domain is a histone-like domain highly positively charged, containing various AAKP tandem repeats, similar to eukaryotic histone H1 and AlgP from *P. aeruginosa,* and is responsible of nonspecific binding to DNA (Maestro et al. 2013). This model suggests that in the absence of PHAs, the amphipathic α-helical N-terminal domain of PhaF presents a partially disordered state although when the polymer is produced, this hydrophobic N-terminal domain interacts with PHA, whereas the hydrophilic two domains are exposed to aqueous cytoplasm and thus structuring the proteinaceous surface layer of the granule (Galán et al. 2011; Maestro et al. 2013). Both the N-terminal and the C-terminal domains have shown to be essential for biosynthesis and the efficient accumulation of PHAs inside the cell. In *P. putida* KT2442, the lack of either of the two domains causes the accumulation of PHAs to decrease appreciably (Galán et al. 2011; Dinjaski and Prieto 2013).

PhaF has been shown to be involved in the organization of the granules along the long axis of the cell and to organize granule sharing during cell division. PhaF binds to DNA nonspecifically through the C-terminal histone-like domain and plays a key role in intracellular localization and equal distribution of carbonosomes to daughter cells during cell division (Sandoval et al. 2007; Galán et al. 2011; Maestro et al. 2013). Furthermore, PhaF might or, putatively, could interact with protein complexes from elongasome or divisome, facilitating the formation of this arrangement by direct or indirect interaction with the cell nucleoid. However, the precise mechanism by which PHA granules are positioned by PhaF remains elusive. An indication of the potential role of PhaF interacting with elongasome or divisome has been recently provided when *phaF* gene was overexpressed in *P. putida* N. recombinant strain acquiring a strong resistance to high ampicillin concentrations at the time that showed an unnatural filamentous morphology (Obeso et al. 2015a).

Moreover, the role of PhaF in the balanced distribution of PHA granules during cell division has been shown via its interaction with the bacterial chromosome (a possible granule carrier during cell division). Importantly, a strain lacking PhaF with an unbalanced granule distribution during cell division shows two markedly different cell populations: those that have and those that lack PHA granules (Galán et al. 2011). Additionally, it has been also proposed that PhaF could be involved in the transcriptional regulation of the *pha* gene cluster (Prieto et al. 1999). However, this point is controversial, and the information on this is contradictory between the strains studied. Thus, first evidence of PhaF involved in the transcriptional regulation of PHA biosynthetic genes was provided n *P. putida* GPo1. In this strain, disruption of the *phaF* gene leads to increased expression of *phaC1*. These results suggested that PhaF acts as a negative regulator of *phaC1* expression in this strain (Prieto et al. 1999). Conversely, the knocking out of *phaF* in *P. putida* KT2440 also affected the transcription of *phaC1*, but significatively decreasing its transcriptional level, and delaying the higher transcriptional level of *phaI*, to a later stationary phase. Concomitantly, disruption of *phaF* also affected PHA production producing a 1.5-fold lowering of the accumulated polymer (Galán et al. 2011).

However, using in vitro interaction/pull-down experiments combined with mass spectrometry, it has been shown that interaction between PhaF and PhaD did not mediate for a third protein. As previously indicated, PhaD is the only protein encoded in *pha* cluster that is not associated directly to carbonosomes but in solution in cytoplasm. In the binding of PhaD to specific promoters controlling expression of *pha* genes, the binding of PhaF has been observed, suggesting that the binding of these two proteins could be modulating the regulatory function of PhaD (Tarazona et al. 2020). The association between these two proteins correlated with alterations observed in transcriptional level alterations of a defective *phaF* mutant derived from *P. putida* KT2440 (Galán et al. 2011; Tarazona et al. 2020).

Interestingly, PhaI phasin and the amphipathic N-terminal region of PhaF share a great similarity level (about 55%) in pseudomonads, including the putative oligomerization linker, which allows for the possibility of heterodimers/heterotetramers formation between both phasins (Tarazona et al. 2019, 2020). Moreover, it has been experimentally shown that the function of PhaI in terms of PHA production can be substituted by PhaF or its N-terminal domain. The PhaF C-terminal domain is, however, essential (Moldes et al. 2004; Maestro et al. 2013). Considering the analogous structure of PhaI to N-terminal domain of PhaF, anchorage of this phasin to the granule could happen in a similar way to PhaF, being crucial for granule structuration and formation (Maestro et al. 2013). The influence on and possible cooperative function of PhaI in determining the homogeneity of the cell population (in terms of granule content and segregation) remains poorly understood (Dinjaski and Prieto 2013).

From a biotechnological point of view, N-terminal domain of PhaF, denominated BioF, has been used as a polypeptide tag to anchor fusion proteins to PHA granules. Taking into account the similarity of PhaI with this domain, this phasin can also be used as a putative tag. Granules expressing these engineered fusion proteins could be easily purified by a simple centrifugation step, and proteins would subsequently be released by treatment with mild detergents, maintaining their enzymatic activity (Moldes et al. 2004, 2006; Dinjaski and Prieto 2013; Mato et al. 2020).

21.4 Biosynthetic Pathways for Monomer Synthesis

The specific strain ability to accumulate scl-PHAs and/or mcl-PHAs are based on differences in the in vivo PHA synthesis machinery, mainly PhaC specificity, which articulates the pathways and hierarchizes the metabolism used for monomer synthesis, as a result of a complete adaptation to the environment and the nutritional traits on it, where these organisms live (Zinn and Hany 2005). This fact results in a prioritization of metabolic fluxes through specific pathways for the maintaining of the cellular fitness at the time that PHA accumulation occurs. Undoubtedly, these metabolic carbon fluxes should be completely under control of specific regulatory systems acting coordinately at whole cell level. Furthermore, metabolic flux of 3HA-CoAs could be one of the supporting carbon sources for the maintaining of bacterial growth and fitness, initiating the formation of PHA inclusions at the time that influences PHA composition. Thus, the knowledge of the biosynthetic pathways driving to the formation of specific monomeric units has allowed for the development of metabolic engineering strategies aiming at tailor-made PHAs.

To sum up, in the biosynthesis of PHB by *C. necator,* this process starts with the condensation of two molecules of acetyl-CoA, forming acetoacetyl-CoA because of the activity of 3-ketothiolase (*phaA* gene). NADP-dependent acetoacetyl-CoA is then stereospecifically reduced by an NADP-dependent acetoacetyl-CoA reductase (encoded by *phaB*) producing the (*R*)-3-hydroxybutyryl-CoA monomer. Finally, the 3-hydroxybutyryl-CoA monomers are added to the growing polymer by a PHA synthase (*phaC* gene) forming the long linear polyester chain of PHB and free CoA (Schubert et al. 1988; Peoples and Sinskey 1989a; Madison and Huisman 1999). These genes are organized in the *phaCAB* operon. This operonic organization can be found in other PHB producers although in other bacteria, a different gene order can be observed. However, as a pattern, *phaC* gene is usually placed within the same locus as *phaA* and *phaB* genes (Schubert et al. 1988; Peoples and Sinskey 1989a, b).

Acetyl-CoA which would be used for PHB (and other scl-PHAs) could be originated through metabolism of sugars, fatty acids, amino acids, or other unrelated molecules. Sugars require a previous conversion to pyruvate through the glycolytic pathway and further into acetyl-CoA, whereas β-oxidation of fatty acids originates acetyl-CoA as end product. Condensation of one molecule of acetyl-CoA with one of propionyl-CoA results in formation of 3-ketovaleryl-CoA, and then proceeding through the classical route for synthesis, (*R*)-3-hydroxyvaleryl-CoA is synthesized and would be polymerized into copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate). Unlike acetyl-CoA, a central metabolic intermediate, propionyl-CoA is only synthesized from a limited number of substrates, such as propionate, fatty acids with an odd number of carbon atoms, and branched chain amino acids (Steinbüchel and Lütke-Eversloh 2003).

Most of *Pseudomonas* species which accumulate mcl-PHAs use two different central pathways for 3HA-CoA obtaining as polymerizable monomers that are to be used by PHA synthetases to biosynthesize PHA: fatty acid de novo biosynthesis pathway or *β*-oxidation pathway (Huijberts et al. 1994). The use of one or the other depends on the structural relatedness of the substrates (carbon sources) used for precursor 3HA-CoA biosynthesis: fatty acids (or related molecules) for the first pathway or nonfatty acid precursors, for example, carbohydrates (Rehm 2003). The monomeric composition of PHAs is strictly influenced by the carbon source used by the strain. In presence of carbon sources structurally related to the synthesized monomer (e.g., fatty acids), the final composition of the polymer reflects that of the substrate. When unrelated substrates (e.g., carbohydrates) are used, the monomeric unit from PHAs are independent of the structure of the carbon source.

21.4.1 β-Oxidation-Derived Monomers for mcl-PHAs

When pseudomonads are cultured using fatty acids and related molecules as carbon sources, *β*-oxidation pathway plays an important role in providing intermediates for mcl-PHA synthesis (Fig. 21.6) (Madison and Huisman 1999). Catabolism of exogenous fatty acids starts with the transport across the membrane. This mechanism has been described as facilitated by FadL in *E. coli* and other bacteria (Nunn et al. 1986;

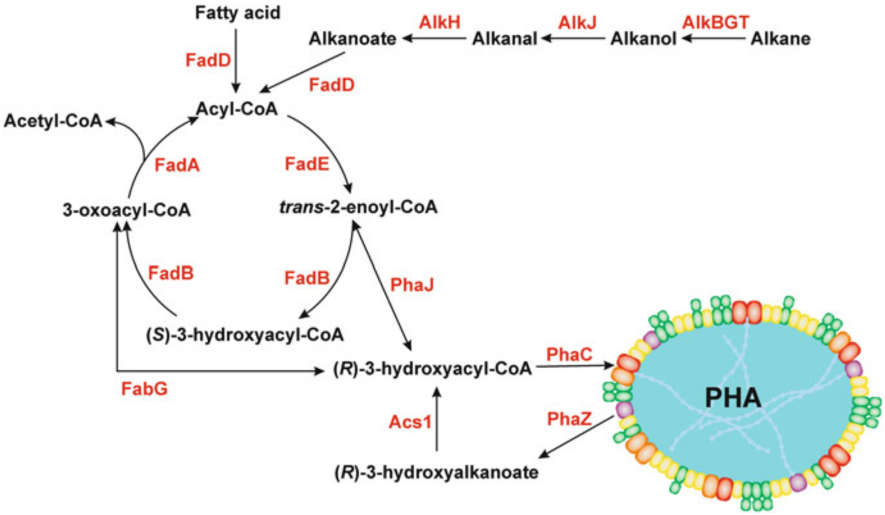

Fig. 21.6 Relationship between β-oxidation of fatty acids and PHA biosynthesis and mobilization. Convergence in this pathway of alkanoate metabolism is also indicated

Black 1988). Although potential *fadL* homologs have been identified in the genome of several pseudomonads, their role in fatty acid transport has not been elucidated (Baker et al. 2018). However, the uptake mechanism of octanoate in *P. putida* U has been reported as constitutive and energy-dependent. It is strongly inhibited by structural analogs of octanoic acid by various fatty acids with a carbon length higher than C5 and by certain phenyl derivatives (Carnicero et al. 2006). At the same time of fatty acids uptake, they are activated to fatty acyl-CoA by acyl-CoA synthetases (AMP forming) (FadD) at the expense of ATP, CoA, and Mg^{2+} (Fig. 21.6) (Kornberg and Pricer 1953; Black et al. 1992). This coenzyme A thioesterification of the fatty acids facilitates their transport across the inner membrane into the cytosol (Weimar et al. 2002). Thus, the vast majority of transported fatty acids in the cytoplasm are in the form of activated acyl-CoA thioesters where they are catabolized through the *β*-oxidation pathway.

In *P. putida* U, two in tandem genes, *fadD1* and *fadD2*, codified fatty acyl-CoA synthetases. However, FadD1 appears to be the main one responsible for activation of both medium-chain length alkanoates and phenylalkanoates since this bacterium can catabolize them and also incorporate both kinds of intermediates to PHA. On the other hand, FadD2 is a cryptic redundant activity of FadD1 which is only expressed under stress conditions when FadD1 is absent (Fernandez-Valverde et al. 1993; García et al. 1999; Olivera et al. 2001a). A similar organization of the two genes encoding fatty acyl-CoA ligases has been found in the genome of *P. aeruginosa* PAO1. However, in these bacteria, FadD1 has a substrate preference for long-chain fatty acids while FadD2 prefers shorter-chain fatty acids (Kang et al. 2010). Moreover, in the genome of this strain, analysis of four homologue genes to *fadD1* and *fadD2* was analyzed, and *fadD4* was identified as codifying an acyl-CoA synthetase involved in citronellate and geraniate, although this protein can also contribute to fatty acid catabolism (Zarzycki-Siek et al. 2013). In *P. putida* GPo1, there are also two acyl-CoA synthetases (ACS1 and ACS2) although in these bacteria, ACS2 has been found associated to cell membrane and is responsible for the activation of exogenous fatty acids, whereas ACS1 is associated to carbonosomes probably being involved in PHA mobilization (Ruth et al. 2008). In *P. putida* KT2440, a third gene, *fadDx,* has been described although its function remains unknown (Wang and Nomura 2010). On the other hand, in *P. putida* CA-3, there is a unique copy of *fadD*, and the acyl-CoA synthetase from this bacterium is responsible of activation of both aromatic and aliphatic alkanoates. Deletion of this gene handicapped this strain for growth and PHA accumulation with 10-phenyldecanoate, decanoate, and longer-chain substrates. Moreover, overexpression of this gene increased 1.6-fold PHA accumulation (Hume et al. 2009).

Once fatty acids have been activated as acyl-CoA derivatives, the catabolism of these molecules requires the participation of an acyl-CoA dehydrogenase (FadE) to start *β*-oxidation cycle. This enzyme catalyzes the oxidation of acyl-CoA to *trans*-2-enoyl-CoA, promoting the α,β-dehydrogenation of these acyl-CoA esters through the transfer of two electrons to the FAD cofactor (Fig. 21.6) (Black and DiRusso 1994). Reaction catalyzed by FadE presents the lowest activity among all *β*-oxidation enzymes and therefore is the rate-limiting step in this catabolic process (Qi et al. 1998; Lu et al. 2003). Bacterial genomes encode several acyl-CoA dehydrogenases although only some of them seem to participate in fatty acid catabolism. Thus, in the genome of *P. putida* KT2440, an orthology group contains 21 genes putatively encoding acyl-CoA dehydrogenases although only a few of them have been experimentally characterized: PP_2216 encodes an acyl-CoA dehydrogenase with high specificity for short-chain acyl-CoA substrates (butyryl-CoA, valeryl-CoA, and hexanoyl-CoA) (McMahon et al. 2005); PP_2437 encodes an acyl-CoA dehydrogenase showing preference for substrates ranging from 10 to 14 carbon atoms, showing clear preference for dodecanoyl-CoA, and their specific mutation impaired, although no completely abolished, growth and PHA accumulation when fatty acids in the range are used as carbon sources (Guzik et al. 2014b); an acyl-CoA dehydrogenase, PP_0368, found as participant in phenylacetate catabolic pathway (Olivera et al. 1998; Luengo et al. 2001), also shows activity recognizing palmitoyl-CoA and stearoyl-CoA as substrates (McMahon and Mayhew 2007). On the other hand, several of the putative acyl-CoA dehydrogenases found in the genome of *P. putida* KT2440 has been identified as involved in other metabolic processes (amino acid or organic sulfur metabolism) not related to fatty acid catabolism (Guzik et al. 2014b).

β-oxidation pathway continues with the hydration of the *trans*-2-enoyl-CoA to (*S*)-3HA-CoA mediated by an enoyl-CoA hydratase activity and with the further oxidation of the hydroxyl group to a keto group, catalyzed by an NAD-dependent 3-hydroxyacyl-CoA dehydrogenase activity, producing 3-oxoacyl-CoA (Fig. 21.6). Both activities, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, are found in a same protein, FadB (Yang et al. 1988). Last step of *β*-oxidation is the cleavage of 3-oxoacyl-CoA to yield acetyl-CoA and an acyl-CoA molecule that has been shortened by two carbon atoms, catalyzed by the 3-ketothiolase FadA (Fig. 21.6); the remaining acyl-CoA enter the next cycle at FadE level (Feigenbaum and Schulz 1975; Imamura et al. 1990). In pseudomonads, genes encoding these proteins are usually arranged as a single *fadBA* operon, and the encoded proteins form a $\alpha_2\beta_2$ tetrameric complex located in the cytosol (Olivera et al. 2001a; Fiedler et al. 2002).

The FadB protein, in addition to the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase enzymatic activities, also has a Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase activity necessary for the degradation of fatty acids with unsaturations located on the odd carbons of the carbonate chain. This activity isomerizes the double bond from the odd carbon to the previous even carbon resulting in an intermediate suitable for continuing β-oxidation cycle (Pramanik et al. 1979; Clark and Cronan 2005). It has also been proposed that the degradation of fatty acids having *cis*-double bonds at even-numbered carbons requires an additional activity in the FadB protein, a 3-hydroxyacyl-CoA epimerase, that would catalyze the transformation of (*R*)-3HA-CoA to (*S*)-3HA-CoA and vice versa. This activity has been suggested in mammalian and plant peroxisomes and bacteria (Chu and Schulz 1985; Yang et al. 1986; Behrends et al. 1988). This epimerase activity could be a very interesting function providing PHA biosynthetic monomers since it could allow us to produce (*R*)-3HA-CoAs from (*S*)-3HA-CoAs produced during β-oxidation. However, this activity is not functional, or at least, its activity is so low in *P. fragi* and *P. putida* that do not contribute to the providing of polymerizable precursors (Imamura et al. 1990; Allenbach and Poirier 2000; Fiedler et al. 2002).

The main physiological role of the *β*-oxidation pathway is the catabolism of fatty acids to produce acetyl-CoA (even propionyl-CoA if the fatty acid has an odd number of carbon atoms) and reducing equivalents (Schulz 1991). These reducing equivalents will be reoxidized using a system coupled to the introduction of electrons to the respiratory electron transport chain associated to plasmatic membrane, allowing for the creation of a proton gradient originated by the movement of these electrons through the components of the chain. The recovery of this chemoelectrical gradient through the membrane allows for the production of metabolic energy supporting growth.

An operonic unit *fadBA* has been characterized in *P. putida* U. Encoded complex FadBA is responsible for β-oxidation of both mcl-alkanoates (from hexanoate to dodecanoate) and phenylalkanoates (arylalkanoates, from 5-phenylvalerate to 10-phenyldecanoate). However, when these *fadBA* genes were mutated, after 80 h of culture in a medium containing alkanoates as sole carbon source, the mutant strains were able to grow. However, if n-phenylalkanoates were used as carbon source, after 800 h of culture, the strains were unable to start the growth. This implies the existence of other genes and proteins that allow β-oxidation of alkanoates but that are unable to metabolize n-phenylalkanoates. Other *fadBA* operons have not been found in the genome of *P. putida* U though different monocistronic genes and operonic units potentially involved in β-oxidation have been observed (Olivera et al. 2001a, b).

In *P. putida* KT2440 and their derivative KT2442, two sets of *fadBA* genes have been described, *fadBA* and *fadAxBx*. Induction of *fadB* is carried out by laureate, whereas this fatty acid is unable to induce *fadBx* gene. This fact suggests that *fadBA* are the genes encoding the canonical β-oxidation, whereas *fadBx* and *fadAx* might participate in fatty acid catabolism only in the absence of functional FadB and/or FadA. No evidences on the direct participation in β-oxidative process has been reported yet (Liu and Chen 2007; Wang and Nomura 2010). Genome of *P. aeruginosa* PAO1 harbors five *fadBA* paralogs, named *fadBA1, 2*, *3, 4*, and *5*. However, among these, *fadBA1 and fadBA5* play major roles in the bacterial fatty acid degradation. The *fadAB1* (PA1736 and PA1737) operon was shown to be strongly induced by medium-chain fatty acids (C10-C2) although it is also induced although to a lesser extent by some long-chain fatty acids (C14-C18) (Son et al. 2008). On the other hand, the *fadBA5* (PA3014 and PA3013) operon was determined to be specifically involved in long-chain fatty acids metabolism and to be induced mainly by oleate and also by other long-chain fatty acids (Kang et al. 2008).

The link between β-oxidation and PHA biosynthesis comes from the formation of the precursors to be polymerized into mcl-PHAs by PHA synthase, (*R*)-3HA-CoAs, from the corresponding intermediates of fatty acid β-oxidative process. For this, three different possible mechanisms have been proposed in pseudomonads: (i) kidnapping of *trans*-2-enoyl-CoA intermediates by a (*R*)-stereospecific enoyl-CoA hydratase (PhaJ) catalyzes the (*R*)-specific hydration of these intermediate metabolites to (*R*)-3HA-CoAs; (ii) epimerization of (*S*)-3HA-CoAs is transformed into (*R*)-3HA-CoAs by an epimerase, and (iii) 3-oxoacyl-CoAs are recognized by a 3-ketoacyl-CoA reductase (FabG), leading to the synthesis of (*R*)-3HA-CoAs (Fig. 21.6). Taking into account the cyclic nature of *β*-oxidation pathway and the shortening of the intermediates in each round, a cocktail of monomeric PHA precursors is generated from this pathway, starting from 3-hydroxyhexanoate toward monomers with longer side chains.

As previously indicated and as far as we know, (*S*) to (*R*) epimerization of 3HA-CoAs from an epimerase activity in FadB has not been proved in pseudomonads (Imamura et al. 1990; Allenbach and Poirier 2000). However, specific hydration of *trans*-2-enoyl-CoA to (*R)*-3-hydroxyacyl-CoA catalyzed by a (*R)*-enoyl-CoA hydratase (PhaJ) has been documented in several *Pseudomonas* spp. (Fig. 21.6) (Tsuge et al. 2000, 2003; Fiedler et al. 2002). There are four isoforms of *phaJ* present in the genome of *P. aeruginosa* DSM1707*, phaJ* to *phaJ4*. PhaJ1 showed a substrate specificity toward enoyl-CoAs with acyl chain lengths from C4 to C6, while PhaJ2-PhaJ4 exhibited substrate specificities toward enoyl-CoAs with acyl-chain lengths from C6 to C12 (Tsuge et al. 2000, 2003; Davis et al. 2008). In *P. putida* KT2440, three *phaJ* genes have been found. These genes have been named *phaJ1, phaJ3,* and *phaJ4* based on similarity of the encoded proteins to the corresponding PhaJs from *P. aeruginosa*. In *P. putida* KT2440, PhaJ4 has been identified as the main (*R*)-enoyl-CoA hydratase participating in the production of (*R*)-3HAs from the β-oxidation intermediates *trans*-2-enoyl-CoAs (Wang and Nomura 2010; Sato et al. 2011). On the other hand, PhaJ1 has a broader substrate specificity than the homologous protein from *P. aeruginosa* since the protein from

P. putida shows tolerance toward short- and medium-chain length enoyl-CoAs (Sato et al. 2011). However, proteins encoded by *phaJ1* and *phaJ3* appear to have little, if any, impact on PHA production on strains KT2440 and LS46 as their level of transcription was much lower than *phaJ4* under PHA production conditions (Wang and Nomura 2010; Fu et al. 2015).

In addition to PhaJ, FabG has also been proposed to link β-oxidation to mcl-PHA synthesis catalyzing the reduction of 3-oxoacyl-CoAs formed during fatty acid catabolism. FabG, belonging to the ketoacyl reductase family of proteins, is an essential enzyme for de novo synthesis of fatty acids in pseudomonads. The canonical reaction for FabG is the catalysis of the NADPH-dependent reduction of 3-oxoacyl-ACP to (*R*)-3-hydroxyacyl-ACP (Lai and Cronan 2004). However, this enzyme can recognize as substrates both 3-oxoacyl-ACPs and 3-oxoacyl-CoAs (Ren et al. 2000b). In cells grown in the presence of fatty acids, FabG kidnaps 3-oxoacyl-CoA intermediates from the β-oxidation pathway to produce (*R*)-3-hydroxyacyl-CoA substrates, which are subsequently incorporated into a PHA polymer by PHA synthase (Fig. 21.6). Thus, main studies on the relationship of this protein with mcl-PHAs have been made with transgenic strains expressing this enzyme. Thus, previous studies have shown that coexpression of the *fabG* genes from *E. coli* and *P. aeruginosa* with PHA synthase genes from different origins enabled recombinant *E. coli* to accumulate mcl-PHA copolymers or even scl-mcl-PHAs when grown in the presence of excess fatty acids such as decanoate or dodecanoate (Taguchi et al. 1999; Ren et al. 2000b; Park et al. 2002; Nomura et al. 2008).

Considering the capital meaning of *β*-oxidation pathway providing PHA precursors from fatty acids and related molecules, many researches have focused on the inhibition of *fadB* and/or *fadA* in producer strains to increase mcl-PHA accumulation and synthesize novel functionalized mcl-PHA (Fig. 21.7). Moreover, modification of this pathway has been shown to be a good strategy for tailoring PHAs since controlling carbon flow through this pathway and using the appropriate carbon sources, PHAs, can be rationally designed to have monomers with a chain length of a desired size or to include organic functions in their chains (Olivera et al. 2001b; Escapa et al. 2011; Chung et al. 2011). As a first approach, chemical inhibition of *β*-oxidation pathway has been performed. For this, sodium acrylate has been widely used. Thus, acrylate blockage of *β*-oxidation pathway in *P. putida* KT2440 resulted in an increase of PHA production as well as an enrichment in monomer contents of 3-hydroxyoctanoate and 3-hydroxydecanoate in mcl-PHA when cultured with decanoate and sodium acrylate (Gao et al. 2018) or 3-hydroxynonanoate and 3-hydroxyheptanoate when cultured in a nonanoic acid-limited chemostat (Jiang et al. 2012). Similar approaches have been successfully performed for improvement of PHA biosynthesis in transgenic microorganisms (Qi et al. 1998).

Other strategy to obtain strains with a weakened *β*-oxidation consists of generating the specific knocking out of *fadB, fadA,* or both genes simultaneously. This approach has been carried out in several strains: *P. putida* U (Fig. 21.7) (Olivera et al. 2001a, b), *P. putida* KT2442 (Ouyang et al. 2007; Liu and Chen 2007), *P. putida* KCTC1639 (Vo et al. 2007), *P. entomophila* L48 (Chung et al. 2013),

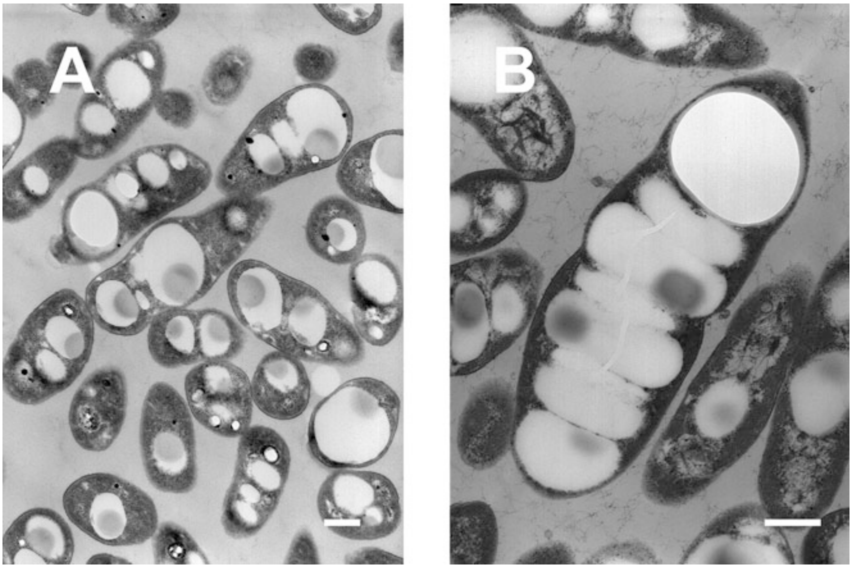

Fig. 21.7 Effect of β-oxidation in mcl-PHA accumulation in *P. putida* U. (**a**) Transmission electron microscopy of wild-type strain. (**b**) Transmission electron microscopy of *P. putida* Δ*fadBA*, a derivative of *P. putida* U in which *fadBA* operon was deleted. Both strains were cultured in a minimal medium containing 4-hydroxyphenylacetate (10 mM) to support growth and octanoate (10 mM) as source of PHA precursors. Bars indicate 500 nm

P. mendocina NK-01 (Zhao et al. 2020), and others. The resulting mutants of the different studied strains showed a phenotype in which, in cultures established with co-feeding strategies, using alkanoates or analogs of these as source of precursors and another carbon source to maintain bacterial growth usually leads to an increase of the content of the synthesized polymer inside the cells. Moreover, through variation of the sources of precursors, tailoring of specific polymers could be made allowing for the obtaining of PHAs with desired monomeric composition and thus with specific physicochemical properties.

Some pseudomonads are also able to synthesize mcl-PHAs used as source of precursors at the time of carbon source, *n*-alkanes (Witholt et al. 1990). Octane and hexadecane are the alkanes most widely used as carbon and energy sources by pseudomonads. For their use, an alkane hydroxylase catalyzes the oxidation of n-alkanes to alkanols (Chen et al. 1995). Alkane hydroxylase of *P. putida* GPo1 consists of three components: an alkane monooxygenase, AlkB, which belongs to a large class of membrane-bound nonheme diiron monooxygenase, and two electron transfer proteins, a rubredoxin encoded by *alkG* and a rubredoxin reductase, AlkT (Kok et al. 1989; van Beilen et al. 1994). This enzyme can recognize linear alkanes and alkenes as substrates (in a hexane to dodecane range) to yield primary alcohols (Nieboer et al. 1993). These alkanols are further oxidized by alkanol dehydrogenases (AlkJ) leading to aldehydes, which are recognized by aldehyde dehydrogenases (AlkH) producing carboxylic acids, which are metabolized through β-oxidation leading to the synthesis of PHA precursors (Fig. 21.6) (Kok et al. 1989; van Beilen et al. 1992).

Interestingly, polymerization and depolymerization of PHA, and therefore biosynthesis and catabolism of monomers, occur simultaneously and are connected through a continuous metabolic cycle, resulting in a constant turnover of PHA (Ren et al. 2009a, 2010; de Eugenio et al. 2010a; Arias et al. 2013). In that sense, PhaZ depolymerase is continuously releasing 3-hydroxy fatty acids from polymers, which are then thioesterified to CoA derivatives by FadD/Acs1 acyl-CoA synthetases (Fig. 21.6), entering β-oxidation pathway for the obtaining of energy or being reincorporated to the polymer by PHA synthase constituting a futile cycle in which the only fact observable is the consumption of ATP giving place to AMP. However, this could also be viewed as a system for metabolic flux regulation between β-oxidation and PHA production. Thus, PhaC synthase is sensitive to the ratio of [(R)-3HA-CoA]/[CoA], with free CoA acting as a moderate competitive inhibitor of PhaC. On the other hand, FadBA, the β-oxidation complex, which acts as a source of 3HA-CoA from fatty acids, is affected by the [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios; thus, high ratios boost accumulation of 3HA-CoAs, whereas low ratios suppose oxidation of 3HA-CoA. To sum up, PHA accumulation is regulated both by [acetyl-CoA]/[CoA] and [NADH]/[NAD] (Ren et al. 2009a, 2010; de Eugenio et al. 2010a).

Linear and branched n-alkanes can also be used as precursor for mcl-PHAs being channeled to β-oxidation (Lageveen et al. 1988; Preusting et al. 1993). For this, *Pseudomonas* need to use alkane hydroxylases (AHs), which catalyze the first step of alkane biodegradation and are the key enzymes in aerobic degradation of alkanes by bacteria. These enzymes hydroxylate alkanes to alcohols, which are further oxidized to fatty acids and catabolized via the bacterial β-oxidation pathway (van Beilen et al. 2001; Rojo 2009). However, these compounds show a limitation in the commercial production of mcl-PHAs due to the limitations derived from two-phase fermentation systems required (Schmid et al. 1998). Precursors structurally related to fatty acids, metabolized through a β-oxidative process, can be incorporated into mcl-PHAs depending on the broad substrate specificity of PHA synthases from pseudomonads. The structures of the precursors used are recovered in the monomers polymerized as PHA (Olivera et al. 2010). Thus, mcl-PHAs containing unsaturated monomers have been obtained from different *Pseudomonas* when cultured in media containing *n*-alkenes, *n*-alkynes, unsaturated fatty acids, triglycerides, and animal or vegetal oils. Thus, when 7-octenoate, 8-nonenoate, or 9-decenoate, or compounds metabolized to or containing these compounds, are supplied as carbon source, PHAs containing derived β-oxidation monomers of these 3-hydroxy-5-hexenoate, 3-hydroxy-7-octenoate, 3-hydroxy-8-nonenoate, and 3-hydroxy-9-decenoate can be obtained (Lageveen et al. 1988; Fritzsche et al. 1990b; Choi and Yoon 1994; Kim et al. 1995b, 1998, 2000; Park et al. 1998; Sparks and Scholz 2008).

Moreover, monomers containing nonterminal unsaturations can also be synthesized using this strategy. Furthermore, this strategy for tailoring PHAs to generate functionalized polymers with new physicochemical properties has been extended to a large variety of organic compounds, methyl-branched alkanoates, and different PHA precursors containing acetylthioesters (Lenz et al. 1992; Scholz et al. 1994b; Ewering et al. 2002; Escapa et al. 2011), acetoxy- (Jung et al. 2000), alkoxy- (Scholz et al. 1994a), cyano- (Lenz et al. 1992), cyclohexyl- (Andújar et al. 1997; Kim et al. 2001; Honma et al. 2004), epoxy- (Bear et al. 1997; He et al. 1998), halogenated- (Doi and Abe 1990; Kim et al. 1992, 1996a; Lenz et al. 1992), or alkylthiol groups (Ewering et al. 2002; Escapa et al. 2011).

Pseudomonas spp. are also able to accumulate PHAs containing aromatic monomers (Olivera et al. 2010; Ishii-Hyakutake et al. 2018). However, availability of aromatic monomers by pseudomonads depends on the adding of aromatic alkanoates as precursors to the cultures. In that way, production of an aromatic monomer was reported for the first time by Fritzsche et al. in 1990, describing a homopolymer of poly(3-hydroxy-5-phenylvalerate) obtained from *P. putida* GPo1 fed with 5-phenylvaleric acid (Fritzsche et al. 1990a). Production of homopolymers and copolymers containing phenylalkanoates from different monomer sizes derived from β-oxidation has also been reported in *P. putida* U (García et al. 1999; Abraham et al. 2001; Olivera et al. 2001a, b). Using a similar strategy, some analogous PHAs bearing phenyl groups have been produced in *P. putida* KT2440, *P. putida* BM01, and *P. citronellolis* (Song et al. 2001; Chung et al. 2001; Mizuno et al. 2014). Incorporation of phenoxy- (Ritter and von Spee 1994; Song and Yoon 1996; Kim et al. 1996b, 2000), methylphenyl- (Hazer et al. 1996; Curley et al. 1996) and methylphenoxy- (Kirn et al. 1999), nitrophenyl- (Aróstegui et al. 1999) or nitrophenoxy- (Kim et al. 1995a), cyanophenoxy- (Kim et al. 1995a; Gross et al. 1996), fluorophenoxy- (Takagi et al. 2004), and thiophenoxy- (Takagi et al. 1999) group containing monomers has also been reported.

21.4.2 De Novo Fatty Acid Biosynthesis as Source of Monomers

In pseudomonads, (*R*)-3HA-CoA precursors for mcl-PHA synthesis can also be synthesized from non-related carbon sources using the de novo fatty acid biosynthetic pathway. This pathway plays a special role in PHA biosynthesis as it can provide various types of 3-hydroxyacyl (3HA) precursors from acetyl-CoA obtained using simple carbon sources such as glucose, fructose, gluconate, glycerol, ethanol, butyrate, and acetate. Thus, carbon sources unrelated to fatty acids are a source of acetyl-CoA: for example, pyruvate dehydrogenase connects glycolytic flux through transformation of pyruvate into acetyl-CoA, and ethanol will be oxidized to acetate which is to be thioesterified with CoA through the action of an acetyl-CoA synthetase (Arias-Barrau et al. 2006). Thus, acetate and acetyl-CoA generated by catabolism of different compounds not related to carboxylates longer than C6 are the precursor molecules for de novo synthesis of fatty acids, leading to the formation of precursors for PHA synthesis. Although 3-hydroxydecanoate is the main monomer found in PHAs synthesized using fatty acid biosynthesis (Haywood et al. 1990; Timm and Steinbüchel 1990; Huijberts et al. 1994), unsaturated precursors (e.g., 3-hydroxy-5-dodecenoate and 3-hydroxy-7-tetradecenoate) are also found in PHAs originated by this anabolic pathway (Huijberts et al. 1992).

De novo fatty acid synthesis is a central metabolic pathway in both eukaryotes and bacteria that carry on through the assembly of two carbons at a time through iterative rounds of Claisen condensations between malonyl-acyl carrier protein (ACP) donor and nascent acyl-acceptor, adding two carbon atoms in each turn (Fig. 21.8). However, the biosynthetic machinery used by eukaryotes and microorganisms for assembling of fatty acids are quite different. Thus, in mammalian, fatty acids are synthesized using a single multisubunit polypeptide that serves as ACP tether and providing the enzymatic activities needed for acyl-chain elongation, the so-called FASI type (Smith 1994; Smith et al. 2003). However, pseudomonads use the dissociated FASII pathway in which multiple discrete enzymes assemble the nascent fatty acid chain on a freely diffusible ACP (White et al. 2005). In the genome of *P. aeruginosa* PAO1, three different genes have been annotated as encoding ACP; however, only *acpP1* (PA2966), which is found in the fatty acid biosynthetic gene cluster, could be used as a substrate for the synthesis of fatty acids (Ma et al. 2017).

De novo fatty acid biosynthesis starts with the synthesis of malonyl-CoA through ATP-dependent carboxylation of acetyl-CoA from bicarbonate (Fig. 21.8). This reaction is catalyzed by acetyl-CoA carboxylase, a multimeric complex which consists of four different proteins: biotin carboxylase (AccC), biotin carboxyl carrier protein (AccB), and a carboxyltransferase made up of two different subunits (AccAD) (Fig. 21.8) (Cronan and Waldrop 2002; Mochalkin et al. 2008). Later, a malonyl-CoA-ACP transacylase (FabD) catalyzes the transthiolation of malonyl from CoA to ACP (Fig. 21.8) (Marrakchi et al. 2002; Chan and Vogel 2010; Nguyen et al. 2014). The next step in the biosynthesis consists of the condensation between acetyl-CoA and malonyl-ACP generating the intermediate 3-oxobutyryl-ACP in a reaction catalyzed by β-acetoacetyl-ACP synthase-type KASIII, FabH, in *Escherichia coli* (Tsay et al. 1992). However, in *P. aeruginosa,* this reaction is catalyzed by the highly diverged KASI/II family 3-oxoacyl-ACP synthase FabY (Fig. 21.8) (Cronan 2006; Yuan et al. 2012b; Six et al. 2014). However, in a mutant strain from *P. aeruginosa* lacking *fabY* gene, a residual initiation of fatty acid synthesis can be observed. It has been proposed that it could in part be due to the 3-oxoacyl-ACP synthase PA3286, a KASIII family condensing enzyme with low catalytic activity when using short-chain acetyl-CoA as an initiating substrate (Yuan et al. 2012a). The main cellular role for PA3286 in wild-type P. aeruginosa is in the shunting of exogenous fatty acid-CoA degradation intermediates from the catabolic β-oxidation pathway into fatty acid de novo synthesis at the octanoyl-CoA substrate chain length through condensation with malonyl-ACP to form decanoyl-ACP (Yuan et al. 2012a).

3-oxoacyl-ACP generated by condensation catalyzed by FabY is then reduced in a NADPH-dependent reaction by a 3-oxoacyl-ACP-reductase (FabG) generating (*R*)-3-hydroxyacyl-ACP (Alberts et al. 1964), which has its hydroxyl group removed by a 3-hydroxyacyl-ACP-dehydratase (FabA or FabZ) leading to the formation of a double bond (Hoang and Schweizer 1997), which is reduced by a NADH-dependent enoyl-ACP-reductase (FabI or FabV) (Fig. 21.8) (Huang et al. 2016). Here, the cycle begins again with the condensation of the resulting acyl-ACP with another malonyl-ACP group, but these successive condensation reactions are catalyzed by either a

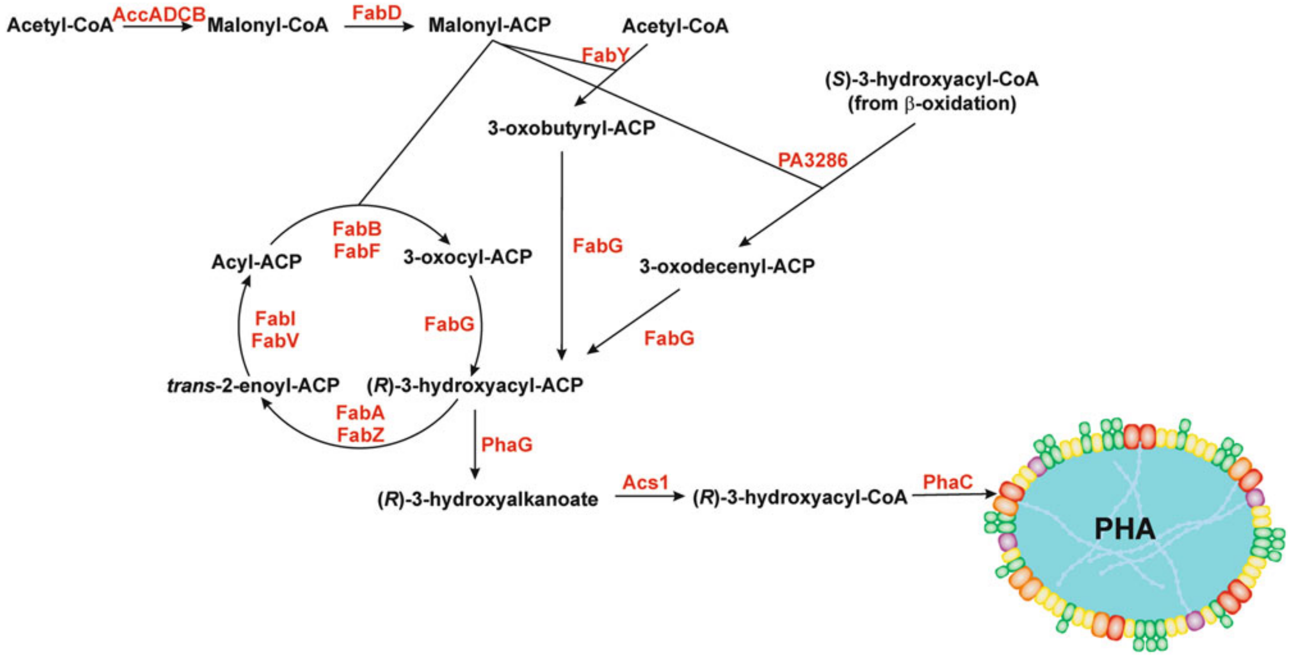

Fig. 21.8 Involvement of fatty acid de novo synthesis and biosynthesis of mcl-PHAs. (Acetyl-CoA is generated through different pathways from glucose, fructose, glycerol ethanol, butyrate acetate, or other compounds)

3-oxoacyl-ACP-synthase I (FabB) (Hoang and Schweizer 1997) or a 3-oxoacyl-ACP-synthase II (FabF) (Mindrebo et al. 2020). The cycle is repeated until the acyl chain reaches 16–18 carbon groups in length, at which point the vast majority of acyl-ACPs are used in membrane biosynthesis.

FabA also participates in the synthesis of unsaturated fatty acids acting as an isomerase on the *trans*-2-decenoyl-ACP, generating *cis*-3-decenoyl-ACP that bypasses the FabI/FabV reduction step (Huang et al. 2016) and can be elongated by FabB (Marrakchi et al. 2002; Zhu et al. 2006). In *P. aeruginosa*, two desaturating enzymes DesA and DesBC, absent from *E. coli*, can also generate unsaturated fatty acids by introducing a double bond in existing phospholipids or saturated acyl-CoAs (Zhu et al. 2006). Contribution of fatty acid de novo synthesis to PHA biosynthesis involves the transformation of (*R*)-3HA-ACP intermediates to (*R*)-3HA-CoA thioesters in a reaction catalyzed by PhaG and is found in most pseudomonads and not co-localized with the PHA biosynthetic cluster (Rehm et al. 1998; Hoffmann et al. 2000, 2002; Fiedler et al. 2000; Leo et al. 2005; Arsad et al. 2009). Although PhaG has been classically considered a specific CoA transferase, it has been shown that PhaG functions as a (*R*)-3HA-ACP thioesterase that produces free (*R*)-3-hydroxyfatty acids from (*R*)-3HA-ACP rather than (*R*)-3HA-CoA (Wang et al. 2012). Therefore, PP_0763 gene product in *P. putida* KT2440 encodes a 3-hydroxyacyl-CoA ligase needed to provide the PHA precursor (*R*)-3-hydroxyacyl-CoA derived from fatty acid biosynthesis into the PHA polymer chain (Wang et al. 2012).

21.5 Regulatory Circuits Involved in PHA Synthesis

Due to the interconnection of PHA biosynthesis with central pathways from pseudomonads, its regulation is quite complex. It involves not only the expression of the PHA biosynthetic machinery but also the regulatory network that should be established between peripheral pathways for the uptake and metabolism of carbon sources, central metabolic pathways, and the interrelationship with biosynthetic machinery. Thus, this complex regulatory network has to act at different levels, from enzymatic regulation by substrates or products and posttranscriptional regulation of these enzymes to transcriptional regulation by specific and global regulatory factors, redirecting the carbon flow from central pathways, β-oxidation or fatty acid de novo synthesis, providing precursors for PHA.

Moreover, differences found in the behavior of different strains of *Pseudomonas* with regard to limitations in the culture growth have been found. Thus, for example, *P. putida* GTo1 or KT2440 seem to be highly influenced for PHA accumulation due to limitations in nitrogen or phosphate in the culture broth. Thus, production of PHAs from fatty acid precursors or by de novo synthesis from unrelated carbon sources depends on a limitation in some of the essential nutrients and an excess of carbon source. However, in a close related strain, *P. putida* U, production of PHAs when the precursors used are fatty acids or phenylalkanoates is not depending on a limitation of nitrogen or phosphorus, being able to accumulate PHAs from an early logarithmic phase of growth (García et al. 1999; Olivera et al. 2001a). However, in this strain, when accumulation is dependent on other sources different of fatty acids (carbohydrates, glycerol, and others) involving fatty acid de novo synthesis, only under a restriction of the availability of nitrogen, PHA accumulation occurs at perceptible level (Humanes, Olivera and Luengo, unpublished results). This fact indicates that regulatory networks affecting either expression or functionality of the PHA biosynthetic machinery, and/or the metabolic pathways involved in precursor providing, are strain dependent, including a level of complexity in the understanding of the mechanisms of global regulation of PHA accumulation.

At first, the simultaneous presence of biosynthetic (PhaC1) and degradative (PhaZ and acyl-CoA synthetase) bound to the carbonosome in *P. putida* GPo1 and in *P. putida* U establishing a futile cycle represents the lower level of regulation of PHA metabolism (Ren et al. 2009a, 2010). This regulation, as above commented, influences activity of PHA synthase at level of ratio of precursor [(*R*)-3HA-CoA] versus free CoA. Moreover, interconnection of PHA metabolism with β-oxidation, which is regulated at level of concentration of acetyl-CoA with respect to concentration of free CoA, involves a mechanism of regulation of metabolic fluxes to and from polymer (Ren et al. 2009a, 2010).

The next level of regulation described in *Pseudomonas*, transcriptional regulation of *phaIF* and *phaC1* by the TetR-like transcriptional activator (PhaD) has been set out above (Klinke et al. 2000; Sandoval et al. 2007; de Eugenio et al. 2010b). Putative participation of PhaF participating in the transcriptional regulation of *pha* genes has been also discussed (Prieto et al. 1999; Galán et al. 2011).

21.5.1 Transcriptional Regulation by RNA Polymerase Specificity

On the other hand, integration of PHA metabolism coordinated within cellular metabolism should involve the participation of global regulators establishing a network of integrative regulatory effects. Initially, a regulatory trait based in selectivity of RNA polymerase controlling transcription on the basis of the use of alternative sigma factors has been described. Thus, as previously commented, in *P. putida* GPo1 and *P. aeruginosa*, two promoters have been reported upstream of the *phaC1* gene, one of them showing consensus sequences for RpoN binding and the other one being dependent on the housekeeping σ^{70} (Huisman et al. 1991; Timm and Steinbüchel 1992). It should be noted that RpoN has been related to nitrogen metabolism and that a limitation on this nutrient triggers PHA accumulation in various pseudomonads. Thus, involvement of this sigma factor on PHA accumulation in *P. aeruginosa* PAK and *P. putida* KT2440 has been investigated by comparing PHA accumulation of wild strains and *rpoN*-negative strains cultured under different concentrations of nitrogen in the media (Hoffmann and Rehm 2004, 2005). In fact, lack of *rpoN* in *P. aeruginosa* impeded PHA accumulation in both gluconate and octanoate, indicating a relationship between this sigma factor and PHA metabolism. Accordingly, PhaG induction in conditions of nitrogen limitation was dependent of RpoN in *P. aeruginosa* but not in *P. putida* (Hoffmann and Rehm 2004). In this way, considering that in *P. putida* KT2440, PhaG is involved in PHA biosynthesis through de novo fatty acid synthesis, lack of RpoN function when the strain was cultured under nitrogen limitation almost duplicated PHA accumulation when octanoate was the source of precursors, although it had no effect on PHA accumulation when the strain was cultured on gluconate (Hoffmann and Rehm 2004; Możejko-Ciesielska et al. 2017).

RpoS, whose levels increase under conditions of nutrient stress or during stationary phase of growth, has been proposed to play a role in PHA metabolism in pseudomonads. To sum up, disruption of *rpoS* gene in *P. chlororaphis* PA23 triggered the downregulation of PHA synthase and phasins coding genes affecting negatively to PHA accumulation in this strain (Sharma et al. 2018). A mutant strain of *P. putida* KT2440 through knockout of *rpoS* was not affected on PHA polymerization, but degradation of polymer when this strain reached stationary phase of growth increased. Thus, after depletion of carbon sources, an increase in the expression driven by the promoter upstream of *phaC1* in the stationary phase was observed although polymer content decreased, indicating that depolymerization was enhanced. These results suggest that transcription driven by promoter upstream of *phaC1* increases the levels of PhaZ when exhaustion of carbon sources is produced. However, the effect of the lack of this sigma factor on a putative promoter upstream *phaZ* gene (Sandoval et al. 2007; Galán et al. 2011) has not been analyzed. Thus, RpoS could participate in the negative regulation of a promoter driving the expression of *phaC1Z*, probably in an indirect way in which PhaD could be acting as an intermediate (Raiger-Iustman and Ruiz 2008).

Moreover, in *P. putida* GPo1, starving and related stressing conditions induce expression of *rpoS,* suggesting a connection between increase of this function and PHA depolymerization in this strain (Ruiz et al. 2004). *P. aeruginosa* PA14 and PAO1 *rpoS* defective mutants showed a significant decrease on PHA accumulation with regard to wild-type strains when cultured using fructose as carbon source. However, when these mutant strains were cultured using decanoate as carbon source and for providing PHA precursors, only the PA14-derived strain showed a similar negative effect. Moreover, *rpoS* inactivation shifted the monomeric composition of accumulated PHAs (Choi et al. 2011).

21.5.2 Co-Regulation of Central Pathways and PHA Metabolism

It is evident that regulation of the central pathways, controlling metabolic fluxes through them, would determine the number of intermediates able for PHA polymerization and, in turn, regulate PHA production and mobilization. Taking this into account, it could be speculated that in the regulatory network controlling PHA content of mcl-PHAs in pseudomonads, regulatory mechanism that govern the interplay of β-oxidation and fatty acid de novo synthesis could be involved in the regulation of PHA biosynthesis and/or degradation. Thus, regulation of fatty acid metabolism has been studied and delineated in *E. coli*. In this bacteria, FadR controls both biosynthesis and degradation of fatty acids. Thus, FadR represses transcription of the genes involved in the catabolism of fatty acids: *fadL*, *fadD*, *fadE*, *fadBA,* and *fadH* in *E. coli* (DiRusso et al. 1993; Black and DiRusso 1994). Furthermore, FadR also has a function as activator of fatty acid de novo biosynthesis upregulating transcription of *fabA*, *fabB,* and *fabHDG* genes (Henry and Cronan 1991; DiRusso et al. 1993; Podkovyrov and Larson 1996; Campbell and Cronan 2001; My et al. 2013).

Moreover, in *E. coli*, unsaturated fatty acid synthesis occurs exclusively by the anaerobic type II biosynthetic pathway and requires the activities of the *fabA* and *fabB* genes. Overimposed to the regulation of these genes by FadR, a repressor protein, FabR, has been described controlling *fabA* and *fabB* expression (Zhang et al. 2002b). Unfortunately, although these regulatory systems seem to be conserved in other γ-proteobacteria (e.g., *Enterobacteriales, Pasteurellales, Vibrionales,* and *Alteromonadales*), orthologs to the encoding genes have not been found in pseudomonads (Kazakov et al. 2009; Subramanian et al. 2010). In *P. aeruginosa* PAO1, it has been documented that PsrA, a member of TetR family of transcriptional regulators, participates in β-oxidation regulation and PHA metabolism as well as in transcriptional regulation of *rpoS* in pseudomonads. PsrA autorepressor acts as a repressor of the *fadBA5* β-oxidation operon (one of the three β-oxidation *fadBA* operons present in this bacteria) and as an activator of *rpoS* (Kojic and Venturi 2001; Kojic et al. 2002; Kang et al. 2008, 2009). Transcriptomics in *P. aeruginosa,* comparing the wild-type and a *psrA*-deficient strain, also showed that the gene *phaC2* is overexpressed in the mutant strain (Kang et al. 2008).

Furthermore, in *P. putida* KT2440, knockout of *psrA* reduced PHA accumulation when using fatty acids as growth and polymer precursor. Moreover, an increase in short-chain length monomers was observed in PHAs accumulated in the *psrA*-defective mutant, suggesting a more active β-oxidation pathway in this mutant strain. Effect promoted by PsrA seems to be a negative regulation of *fadBA* genes in an *rpoS*-independent manner. However, when comparing expression of *fadE* and *fadBx* in both the *psrA* mutant and a *rpoS* defective mutant derived from *P. putida* KT2442, both strains showed a reduced expression of these genes than the wild-type strain, indicating that these genes are regulated by *PsrA* via *RpoS* (Fonseca et al. 2014).

21.5.3 Regulation Mediated by Stringent Response

Stringent response is a broadly conserved bacterial stress response which controls adaptation to nutrient starvation, being activated by several different stress signals (Potrykus and Cashel 2008; Hauryliuk et al. 2015). Taking into account that PHA accumulation in a large group of pseudomonads is triggered off by a nutritional limitation, participation of stringent response in the control of PHA metabolism seemed to have a kind of physiological logic. This response is mediated by the synthesis of alarmones: guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp), together denoted as (p)ppGpp. Alarmones serve as a second messenger signal of nutrient starvation for reprograming transcription of a high set of genes. Alarmone levels are regulated through the enzymes RelA (ppGpp synthetase) and SpoT (ppGpp hydrolase) (Barker et al. 2001a, b; Potrykus and Cashel 2008; Hauryliuk et al. 2015). RelA is typically activated in response to nitrogen and amino acids starvation, whereas SpoT levels respond to phosphate, carbon, or fatty acids starvation (Hauryliuk et al. 2015). In *Escherichia coli,* alarmone ppGpp regulates expression of *rpoS* when this bacterium enters the stationary phase of growth (Gentry et al. 1993). Similar results have been documented in *P. putida* KT2440 (Ankenbauer et al. 2020). A strong correlation between glucose depletion and increasing levels of ppGpp in plug flow reactor cultures of *P. putida* KT2440 has been recently documented (Ankenbauer et al. 2020).

In *P. putida* KT2440, inactivation of both *relA* and *spoT* genes eliminated the need of nitrogen limitation for PHA accumulation using oleate as carbon source, and the mutant, unable to induce the stringent response, showed same level of accumulation independently of nitrogen concentration in the culture broths and also accumulated significatively higher amount of polymer than the wild-type strain under no limitation of nitrogen. Furthermore, in the *relA*/*spoT* mutants, main variation of expression levels of the *pha* genes was observed for *phaI*/*phaF* genes, which were highly upregulated, indicating that stringent response negatively affects the expression of the phasins genes. Significatively, expression of *phaG*, linking fatty acid de novo synthesis with PHA accumulation, was highly increased (Możejko-Ciesielska et al. 2017). In *P. chlororaphis* PA23, mutations in *relA*/*spoT* significantly decreased PHA accumulation, and downregulation of *pha* genes expression was observed. Surprisingly, *phaC2* was significantly upregulated in *relA*/*spoT* mutants. Moreover, a change in the monomeric composition of PHAs accumulated by these mutants when cultured in glucose was observed (Sharma et al. 2018).

21.5.4 Quorum Sensing Related Regulation

Quorum sensing is a mechanism used to standardize gene expression between microorganisms present in a specific niche in response to environmental conditions. This response is mediated through the participation of tiny molecules, called autoinducers, which are synthesized intracellularly and diffuse passively or are actively released out of the cells. Thus, quorum sensing is a microbial cell-to-cell process that relies on the production and detection of autoinducers. In gram-negative bacteria, these autoinducers are acyl homoserine lactones, alkylquinolones, α-hydroxyketones, and diffusible fatty acid-like signal factors (Hawver et al. 2016).

Quorum sensing machinery has also been observed as directly or indirectly associated to the regulatory network controlling PHA metabolism in. *P. chlororaphis* PA23 (Mohanan et al. 2019). Three different quorum sensing systems have been identified in these bacteria: PhzRI, CsaRI, and AurRI (Shah et al. 2020). A derivative of *P. chlororaphis* PA23 expressing a lactonase impedes all quorum sensing signaling in this bacterium, and due to this, the strain is phenotypically equivalent to a mutant defective simultaneously in *phzI*, *csaI,* and *aurI* (Selin et al. 2012). This quorum sensing defective strain showed a significantly reduced

PHA accumulation when using octanoate or glucose as a source of precursors. Moreover, in these bacteria, monomeric composition of PHAs was also altered, and expression of all the genes from *pha* cluster was reduced when cultured in glucose, although when octanoate was the carbon source, all *pha* genes were downregulated except *phaC2* (Mohanan et al. 2019).

Using a random transpositional mutagenesis protocol in *P. putida* CA-3, a mutant strain unable to accumulate PHAs was obtained. In that strain, transposon was disrupting the *gacS* gene, and *trans*-expression of the disrupted gene in the mutant recovered the ability to produce PHAs (Ryan et al. 2013). This gene encodes a sensor kinase protein belonging to a GacS-GacA two-component system that has been considered as part of the quorum sensing machinery in base that its activation occurs when cells grow to high densities, particularly during transition from exponential to stationary phase of growth (Heeb and Haas 2001; Kay et al. 2005). When GacS activates the response regulator GacA, this regulatory protein activates the transcription of *rsmX*, *rsmY,* and *rsmZ* genes, which encode small RNAs which have a high affinity for certain RNA-binding proteins that act as translational repressors, for example, RsmA (Valverde et al. 2003; Heeb et al. 2006; Kusmierek and Dersch 2018).

Surprisingly, in *P. putida* CA-3 mutant strain defective in GacS, transcription of the small RNAs RsmY and RsmZ was not affected. This mutant also showed a lower amount of PHA synthetase although transcriptional level of *phaC1* was maintained, suggesting a translational control of this activity (Ryan et al. 2013). Inactivation of *gacS* in *P. chlororaphis* PA23 also showed a strongly decrease in PHA accumulation. However, in this strain, decrease of PHA synthesis and storage has been linked with a strong downregulation of *phaC1, phaC2, phaD, phaF,* and *phaI*. Moreover, PHA composition in the *gacS* mutant showed a change in monomeric composition (Sharma et al. 2018). In *P. putida* KT2442, inactivation of *gacS* showed a phenotype like *P. chlororaphis* PA23 mutant: diminished amount of stored PHA and reduced level of transcription of the *pha* genes. Moreover, in this strain, restoration of the transcriptional levels of the PHA biosynthetic cluster was achieved through *trans*-expression of *phaD* gene (Prieto et al. 2016).

ANR (anaerobic regulator) is a global regulator belonging to the FNR-CRP (cAMP receptor protein) superfamily (Körner et al. 2003) which is believed to play a key role in allowing bacteria to adapt to microaerophilic or anoxic conditions controlling *hcnABC* transcription and hydrogen cyanide production in *P. aeruginosa*, *P. protegens* CHA0, and *P. chlororaphis* PA23 (Laville et al. 1998; Pessi and Haas 2000; Nandi et al. 2016). Furthermore, genetic control of the genes involved in energy production under low O_2 conditions in *P. aeruginosa*, such as those encoding denitrification pathways, high-affinity cytochrome oxidases, and arginine fermentation enzymes, is carried out by ANR (Zimmermann et al. 1991; Ye et al. 1995). Under water-saturated soil conditions, a *P. protegens* CHA0 *anr* mutant exhibited a 30% decrease in biocontrol ability highlighting the functionality of ANR in soils with restricted oxygen availability (Laville et al. 1998). Nevertheless, in *Pseudomonas putida* KT2440, ANR positively regulates expression of the Cbb3–1 terminal oxidase during exponential growth in highly aerated medium (Ugidos et al. 2008).

Furthermore, inactivation of *anr* gene in *P. chlororaphis* PA23 decreases accumulation of PHAs, and these PHAs, when the mutant strain is cultured with octanoate as carbon source, contain higher C10 monomers than the one obtained from the wild-type strain. Expression of the *pha* genetic cluster was analyzed in this mutant growing on glucose, and only *phaF* showed a significant reduction on its expression. Nevertheless, in upstream from *phaF,* there are no sequences for binding of Anr, suggesting that the phenomena observed depends on an effect mediated by Anr but not through direct interaction of this regulatory protein with *phaF* promoter (Mohanan et al. 2019).

21.5.5 Regulation of PHA Content at Translational Level

21.5.5.1 Catabolite Repression Control

Catabolite repression control protein (Crc) plays a key role in carbon catabolite repression in pseudomonads. This process allows for the preferential assimilation of specific carbon sources among a mixture of several compounds. Crc protein controls gene expression posttranscriptionally, inhibiting the translation of mRNAs containing a Catabolite Activity motif close to the translation star site. The Crc protein shares up to 25 to 32% sequence homology with DNA repair enzymes of other bacteria but does not show any endonuclease or DNA binding ability (MacGregor et al. 1996). Moreover, the crystal structure of Crc from *P. aeruginosa* lacked nuclease activity (Milojevic et al. 2013). Thus, the binding of Crc close the ribosome binding sites occurs in association with another protein, Hfq, and the presence of Crc, Hfq, and the Catabolite Activity motif is essential for Crc regulation. Hfq is an hexameric protein belonging to the class of Sm family of RNA-binding proteins in many bacteria and has a proximal and a distal RNA-binding site (Zhang et al. 2002a; Sauer and Weichenrieder 2011). Thus, Hfq recognizes the "Catabolite Activity" motif in an mRNA and facilitates a Crc-dependent repression of its translation initiation by binding of Hfq-Crc complex near to ribosome binding site (Moreno et al. 2015; Sonnleitner et al. 2018; Kambara et al. 2018).

The strength of Crc-dependent catabolite repression depends on the levels of one or more small RNAs (sRNAs), namely, CrcZ in *P. aeruginosa*, CrcZ and CrcY in *P. putida*, and CrcZ and CrcX in *P. syringae* (Sonnleitner et al. 2012; Moreno et al. 2012; Filiatrault et al. 2013). These sRNAs contain several "Catabolite Activity" motifs, sequestering Hfq and antagonizing Crc, modulating the protein complex formation with mRNA when their effect is not needed, for example, when a poor carbon source is used (Sonnleitner et al. 2009; Moreno et al. 2012, 2015; Milojevic et al. 2013; Sonnleitner and Bläsi 2014). Moreover, the levels of CrcZ and CrcY fluctuate according to the carbon source being used. The transcription of *crcZ* and *crcY* occurs from RpoN-dependent promoters that rely on the CbrA/CbrB two-component regulatory system, which regulates and establishes a healthy carbon/nitrogen balance in *Pseudomonas* (Sonnleitner et al. 2009; Moreno et al. 2012; García-Mauriño et al. 2013). The activity of the promoters controlling the expression of *crcZ* and *crcY* is highest in cells growing at the expense of a poor carbon source such as oxaloacetate, leading to sequestration of Crc which allows for ribosome binding and translation of the mRNAs for utilization of non-preferred carbon source. Furthermore, the activity of these promoters is lowest when cells are cultivated in a complete medium such as LB, whereas when succinate or glucose are provided, the activity of these promoters is intermediate (Valentini et al. 2014; Sonnleitner et al. 2018).

A Crc binding domain in *phaC1* mRNA from *P. putida* KT2442, indicating a role of this protein, controlling the regulatory response to carbon source utilization, with PHA metabolism has been identified. Thus, it has been demonstrated that Crc activity inhibits PHA intracellular accumulation in *P. putida* KT2442 through the inhibition of translation of *phaC1* although not affecting *phaI* or *phaF* translation (La Rosa et al. 2014). Moreover, Crc activity mediating effects over PHA accumulation is influenced by the carbon source used in the fermentation and by the C/N ratio in the culture medium. Accordingly, octanoate elicited a Crc-dependent catabolite repression response in a minimal medium when C/N ratio was balanced although when this ratio was imbalanced, this response could not be detected. Thus, Crc negatively controls PHA synthesis when the bacteria are growing in C/N balanced media. Under C/N imbalanced conditions, CrcY and CrcZ sRNAs levels are increased, sequestering Crc antagonizing its function. Thus, in balanced medium, octanoate activates transcription of *pha* genes, but Crc acts decreasing the levels of PhaC1, redirecting carbon flow for the metabolic utilization for cell growth. Accordingly, in a *crc*-defective *P. putida* KT2442 strain growing in LB supplemented with octanoate, biomass production was lower than in the wild type although PHA accumulation increased (La Rosa et al. 2014).

As previously indicated, transcription of CrcZ and CrcY sRNAs depends on the two-component sensor-regulator system CbrAB (Sonnleitner et al. 2009; Moreno et al. 2012; García-Mauriño et al. 2013) although signals to which CbrA sensor protein responds are still unclear. Thus, it could be a close relationship between this two-component system and PHA accumulation in *P. putida* KT2442, but unfortunately, there is no experimental evidence on this point. To sum up, a deeper understanding of the regulatory networks controlling PHA metabolism and its relationship with global metabolism may facilitate the development of bacterial strains able to accumulate large amounts of PHA when growing using cheap carbon sources, thereby reducing the costs associated with the production of PHAs.

21.5.5.2 Phosphoenolpyruvate: Carbohydrate-Phosphotransferase System

Canonical phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) refers to a group of enzymes and proteins that transfer high-energy phosphate originated in phosphoenolpyruvate from one member of the chain to the next in a stepwise fashion mediating phosphorylation and subsequent uptake of carbohydrates in bacteria. However, these bacterial multiprotein phosphorylation chains have evolved in a regulatory system which integrates different signals (e.g., extracellular carbon and nitrogen sources, endogenous phosphoenolpyruvate levels, physicochemical conditions) triggering posttranslational regulation of target proteins that participate in a multitude of regulatory functions (Cases et al. 2007).

PTS system from *P. putida* KT2440 is formed by two different modules, a fructose intake component encoded by two genes, *fruA* and *fruB*, and a nitrogen-related PTS, involved in regulation of the carbon versus nitrogen balance, and formed by three proteins, EI^{Ntr} (encoded by *ptsP*), NPr (encoded by *ptsO*), and $EIIA^{Ntr}$ (encoded by *ptsN*). FruAB constitutes the only fructose uptake system present in *P. putida* KT2440. The so-denominated nitrogen-PTS branch consists in a phosphotransfer chain initiated from the transference of pyrophosphate from phosphoenolpyruvate to EI^{Ntr}, which in turn phosphorylates NPr and, in final stance, transfer the phosphate to EII^{Ntr} (Velázquez et al. 2007). Interaction of this PTS system with central metabolism in *Pseudomonas* has a direct impact on PHA accumulation in these bacteria. Thus, a direct physical interaction of $EIIA^{Ntr}$ with the E1 subunit (*aceE*) from pyruvate dehydrogenase complex has been published in such a way that phosphorylation state of $EIIA^{Ntr}$ seems to be directly influencing pyruvate dehydrogenase activity and thus modulating acetyl-CoA levels (Pflüger-Grau et al. 2011). This interaction, affecting levels of acetyl-CoA, can justify that in a *P. putida* KT2440 $EIIA^{Ntr}$-defective mutant, this protein is not interacting with pyruvate dehydrogenase, and thus, an increase of acetyl-CoA levels inside the cell is produced, triggering off the accumulation of about 70% more PHAs from octanoate than the wild type. On the other hand, *ptsO* or *ptsP* mutants were almost totally unable to produce PHAs since the loss of NPr or $EIIA^{Ntr}$ results in a greater concentration of non-phosphorylated PtsN and thus inhibiting the pyruvate dehydrogenase, and as a consequence, less acetyl-CoA is produced (Velázquez et al. 2007).

21.6 Drawbacks for Industrial Production of PHAs

Despite the increase of potential applications of PHAs, these compounds have achieved only a limited market penetration, and there is only limited production of PHA worldwide (Choi and Lee 1999; Chen and Patel 2012; Chen et al. 2020). Commercial success has been, until now, mainly frustrated by the high cost of production when compared with petrochemical plastics since PHAs are estimated to be 15 times more expensive than petrochemical plastics (Singh et al. 2009). There are different evident aspects in which it is possible to intervene in order to achieve a competitive industrial production of PHAs which allows for the introduction of these polymers on the market, as goods for consumption, displacing plastics of petrochemical origin. These points refer to the design and use of strains that are able to overproduce the PHAs of interest, that these organisms can grow and produce PHAs from affordable and cheap carbon sources, and that the recovery and purification systems of the polymers are efficient and cheap. Next, the current state of the art in some of these aspects will be pointed.

21.6.1 Use of Inexpensive Carbon Sources

mcl-PHA precursor substrates (commonly pure sugars and/or fatty acids) would account for approximately 50% of production cost of PHAs, negatively affecting the bulk application of PHAs (Koller et al. 2017). Taking this into account, renewable feedstocks have been explored, and fermentative and bioprocesses design are currently being researched for valorization of industrial waste and by-products, with the aim of developing sustainable biotechnological processes for industrial PHAs production. Limited research has been made on mcl-PHA production using molasses. Molasses, an inexpensive and widely available by-product of sugar production, is composed of up to 60% of sugars, containing nearly 45% sucrose, along with simple sugars (mainly glucose and fructose). This limited investigation is due to the fact that the majority of the *Pseudomonas* species are unable to utilize sucrose as carbon source (Solaiman et al. 2006). Thus, to date, only two species of *Pseudomonas* have been shown to metabolize sucrose directly, *P. fluorescens* A2a5 and *P. corrugata* 388.

P. fluorescens A2a5 produced P(3HB) with a yield of 70% cell dry weight when cultured using sugarcane liquor (Jiang et al. 2008). On the other hand, *P. corrugata* 388 was able to consume sucrose from soy molasses producing mcl-PHAs with a yield of 17% cell dry weight (Solaiman et al. 2006). Surprisingly, although *P. mendocina* CH50 was able to accumulate mcl PHA growing in presence of sugarcane molasses, this strain was not able to use the sucrose present in the media (Basnett et al. 2020). PHA production using *Pseudomonas* spp. has been reported using both edible oils and oils without value for human consumption. Research has been mainly performed into plant oils although the use of waste oils from animal origin has also be documented. Ability to hydrolyze triglycerides, and thus to obtain fatty acids that would produce monomers for PHA accumulation, falls to the presence of lipase activities in the strains used. However, some authors have reported that some bacteria showing lipase activity were unable to produce PHAs from some vegetable oils; that drawback was overcome by using an additional saponification step (Silva-Queiroz et al. 2009; Możejko and Ciesielski 2013).

Tallow is a cheap fat that is often produced in quantities exceeding market requirements, making it a relatively affordable carbon source. Thus, different *Pseudomonas* spp. have been assayed to produce PHAs using tallow, either previously hydrolyzed or not. Unfortunately, low PHA yields were obtained, the best results obtained when *P. resinovorans* was cultured with unhydrolyzed tallow, obtaining a 15.2% of PHA content (Cromwick et al. 1996). Pollock (*Theragra chalcogramma*) oil, a by-product of the Alaskan fishing industry, was used to culture *P. oleovorans* NRRL B-14683, *P. resinovorans* NRRL B-2649, *P. corrugata* 388, and *P. putida* KT2442, allowing for the obtaining of mcl-PHAs: 52–53% from *P. oleovorans* strains, 43% in the cultures of *P. corrugata*, and 39% produced by *P. putida* (Ashby and Solaiman 2008).

P. oleovorans ATCC 29347 was reported to be able to use as carbon sources and as source of precursors for mcl-PHAs biosynthesis different from hydrolyzed vegetable oils: olive (26% cell dry weight), hazelnut (60%), and sesame (41%), as well as hamci oil (28%), an oil obtained from a fish, similar to anchovy, taken from the Black Sea (Hazer et al. 1998). It has also been shown that this strain is able to assimilate saponified jatropha oil, a nonedible oil obtained from the plant *Jatropha curcas*, as the sole carbon source to produce a PHA yield of 26% (Allen et al. 2010). Palm oil production originates as residual product of a low-grade oil, rich in free fatty acids, called sludge palm oil. This waste could be used by several pseudomonads to produce mcl-PHAs. *P. putida* S12 has been reported, until now, as the bacterial strain producing the highest yield of mcl-PHAS from this carbon source (Kang et al. 2017).

A culture of *P. putida* PGA1 using saponified palm kernel oil (oil obtained from the kernel of the fruit) as carbon source and for mcl-PHA production resulted in a production ranging 19–37% containing 3-hydroxyoctanoate as the prominent component (Tan et al. 1997). When these cultures were made in a fermenter-scale experiment, 70% of mcl-PHA was obtained at 12 h of fed-batch cultivation (Annuar et al. 2007). *P. mosselii* TO7 cultured with palm kernel oil as carbon source also produced mcl-PHAs consisting of a terpolymer composed primarily of 3-hydroxyoctanoate (43.8%), 3-hydroxydecanoate (36.7%), and 3-hydroxydodecanoate (15.7%) (Chen et al. 2014). *Pseudomonas aeruginosa* cultivated with the oleinic fraction of palm oil was analyzed for the simultaneous production of PHA and rhamnolipids, with an accumulation of 36% mcl-PHAs consisting of a mixture of monomers with 6 to 14 carbon atoms (Marsudi et al. 2008). Copra (coconut) oil has been used to produce mcl-PHAs by *P. guezennei* biovar *tikehau*, producing a 36% of a polymer mainly composed of 3-hydroxyoctanoate and 3-hydroxydecanoate (Simon-Colin et al. 2008). Production of mcl-PHAs from copra oil has also been referred to using *P. mendocina* CH50 as producer. In this bacterium, accumulation observed was 58% of cell dry weight of a terpolymer comprising of 3-hydroxyoctanoate, 3-hydroxydecanoate, and 3-hydroxydodecanoate (Basnett et al. 2018).

Waste cooking oil has been also proposed as potential source of PHAs. Waste cooking oil is abundantly produced around the world, with over 29 million tons produced annually (Maddikeri et al. 2012). Use of this waste potentiates production of PHAs on the basis of its high content in free fatty acids. Thus, this waste cooking oil has been used to produce mcl-PHAs from C6 to C16 by *Pseudomonas* sp. strain DR2 (Song et al. 2008). A study compared PHA production by *Pseudomonas aeruginosa* 42A2 when cultured in waste-free fatty acids from soybean oil, waste frying oil, or glucose. Under these conditions, accumulation from free fatty acids allowed for the accumulation of 66% from soybean oil, 29.4% when waste frying oil was used, and 16.8% when the sole carbon source was glucose. Moreover, although from glucose all the monomers in the PHA were saturated derivatives, unsaturated were found when the waste oils were used (Fernández et al. 2005). Culture of *Pseudomonas* sp. strains GI01 and GI06 using, as precursor for PHA synthesis, waste rapeseed oil obtained after frying gave, as a result, the accumulation of PHA by strain GI01 consisting predominantly of 3-hydroxyoctanoate and 3-hydroxydecanoate monomers. However, the polymer accumulated by the strain GI06 contained significant amounts of 3-hydroxyhexanoate (Możejko et al. 2011;

Możejko and Ciesielski 2014). Furthermore, strain GI01 accumulated up to 43% of cell dry weight (CDW) when using waste palm oil as sole carbon source (Możejko and Ciesielski 2013). Accumulation of high amounts of mcl-PHAs by *P. putida* KT2440 using hydrolyzed waste cooking oil as sole carbon sources growing to high cell density has also been reported (Ruiz et al. 2019).

The main by-product of biodiesel produced by transesterification of a vegetable oil catalyzed by a base is glycerol. It has been calculated that every 100 kg of biodiesel produced generated 10 kg of glycerol. Thus, the fast development of the biofuel industry has generated a considerable amount of crude glycerol (Suppes 2010). Moreover, bioethanol production using *Saccharomyces cerevisiae* as biocatalyst generates glycerol up to 10% of the total sugar consumed in the fermentation (usually sucrose) (Hasunuma and Kondo 2012; Mohd Azhar et al. 2017). As a consequence, glycerol has become a very attractive substrate for bacterial fermentations (Mota et al. 2017). Moreover, to improve the viability of the biofuel economy, the conversion of waste glycerol into value-added products (like PHAs) has become a relevant need (Pagliaro 2017).

Pseudomonads metabolize glycerol through its transformation into dihydroxyacetone phosphate which are transformed into glyceraldehyde 3-phosphate entering the central metabolism via gluconeogenesis and Embden-Meyerhof-Parnas pathway (Lessie and Phibbs 1984; Poblete-Castro et al. 2020b). Final product of this pathway, pyruvate, is then transformed into acetyl-CoA, starting the synthesis of mcl-PHA precursors through fatty acid de novo synthesis (Beckers et al. 2016; Poblete-Castro et al. 2020b).

Thus, *P. putida* GO16 has been reported to be able to produce 6,8 g/L of mcl-PHA (33% of its cell dry weight) after 48 h of cultivation on a fed-batch culture using raw glycerol as carbon source (Kenny et al. 2012). *P. mosselii* TO7 also accumulated mcl-PHAs yielding a 48% of CDW, mainly composed of 3-hydroxyoctanoate (70.6%), when cultured with crude glycerol as sole carbon source (Liu et al. 2018). Furthermore, in a study comparing the abilities of different strains belonging to *P. putida* species, the strain KT2440 resulted in the highest producer of mcl-PHAs (34.5%) using raw glycerol as carbon source. Strains KT2442, F1, and S12, cultured in the same conditions, although also accumulated mcl-PHAs, did so with lower efficiency. Interestingly, during fermentation of strain KT2440, citrate was accumulated at a significative amount (20 g/L) as a by-product in the culture broths (Poblete-Castro et al. 2014). Metabolite flux optimization to avoid accumulation of citrate from tricarboxylic acids cycle could result in an increase of polymerization intermediates improving PHA accumulation from glycerol. Other approaches to engineer more efficient strains to produce PHAs using glycerol as carbon source have been performed, as, for example, knocking out *glpR,* the gene encoding the transcriptional regulator directing uptake of glycerol and its transformation in glycolytic intermediates, doubling mcl-PHA content produced by *P. putida* KT2440 in shaken-flask cultivations with glycerol as the carbon source (Escapa et al. 2013); deletion of *phaZ* in this strain resulted in an increase of 1.4-fold of mcl-PHAs (Poblete-Castro et al. 2014). Moreover, integration of transcriptomics analyses and metabolic fluxes in *P. putida* KT2440 and *P. putida* LS46 have suggested several genetic targets to engineer strains with an improved accumulation of PHAs using glycerol as carbon source (Fu et al. 2015; Beckers et al. 2016). When *P. putida* CA-3 was cultured on volatile fatty acids derived from aerobically digested ensiled grass, it produced 39% of CDW of a PHA mainly composed of 3-hydroxydecanoic acid (Cerrone et al. 2014).

Demonstrated metabolic plasticity of bacteria belonging to genus *Pseudomonas* allows them to utilize and degrade a great variety of carbon sources, having been used for the development of several techniques for bioremediation (Nogales et al. 2017a). Therefore, coupling of biodegradative processes as a potential source of precursors for PHA accumulation could represent a double benefit. In this way, xenobiotic-contaminated environments have been used for the isolation of *Pseudomonas* with the ability to produce PHA. For example, it has been recently reported that *Pseudomonas* strains isolated from contaminated soil and oily sludge samples from Iranian southwestern refineries accumulated 20–23% of their CDW to mcl-PHA using 2% v/v crude oil as the sole carbon source (Goudarztalejerdi et al. 2015). *Pseudomonas* sp. TN301, a strain isolated from a river sediment sample close proximity to a petrochemical industry, was analyzed for production of mcl-PHAs using monoaromatic and polyaromatic hydrocarbons that were examined as PHA precursors (Narancic et al. 2012).

P. fluva TY16, isolated from a petroleum-contaminated soil sample, was able to produce 68.5% of PHA when cultured with decanoate as source of precursors. When this strain was cultured using a continuous feeding strategy with benzene, toluene, and ethylbenzene, PHA contents reached up to 19.1, 58.9, and 28.6% respectively (Ni et al. 2010). Furthermore, it has been reported that *P. putida* F1 is able to accumulate mcl-PHAs when supplied with toluene, benzene, or ethylbenzene, reaching 22, 14, and 15% of its CDW, respectively. *P. putida* mt-2 accumulates mcl-PHA when supplied with toluene or p-xylene, with values of about 25% of its CDW (Nikodinovic et al. 2008). Using transgenic derivatives of *P. putida* A514, overexpressing *phaJ4* and *phaC1* or *phaG*, *alkK*, and *phaC1* under different nitrogen concentrations, biotransformation of vanillate to mcl-PHAs has been documented (Wang et al. 2018).

Design of genetically modified derivatives of *P. putida* KT2440 for metabolic engineering allowing for the conversion of *p*-coumarate and lignin or ferulate into PHAs has been recently documented. Bioconversion of *p*-coumarate and lignin was accomplished by knocking out of *phaZ* and genes involved in b-oxidation at the time that *phaG*, *alkK*, and the genes encoding PHA synthases (*phaC1* and *phaC2*) were overexpressed (Salvachúa et al. 2020). For PHA production from ferulate, a CRISPR/Cas9n-based strategy was developed. Using this technology, two genetic modules for ferulate to vanillate and from vanillate to protocatechuate were inserted in the genome, an extra *phaC1* was also inserted, and *tesB* and *tesBII* were deleted, allowing for the production of 0.27 g/L of PHA from 20 mM ferulate (Zhou et al. 2020).

On the other hand, *P. putida* CA-3 accumulated mcl-PHA using, as carbon source, styrene, phenylacetate, and glucose (Ward et al. 2005; O'Leary et al. 2005;

Goff et al. 2007). Effective PHA accumulation from styrene has also been reported for *P. putida* NBUS12 (Tan et al. 2015).

Using *P. putida* CA-3, a strategy for conversion of polystyrene waste into PHAs, has been proposed. Using this method, polystyrene is converted into styrene oil by pyrolysis, and this oil was used to feed the CA-3 strain (Ward et al. 2006; Goff et al. 2007). Using a similar approach, 1.1 g/L from the solid fraction (terephthalate) of pyrolyzed polyethylene terephthalate was used for PHA production using *P. putida* GO16, *P. putida* GO19, and *P. frederiksbergensis* GO23, accumulating 27, 23, and 24% of their cell dry weight as polymer, under nitrogen limitation conditions (Kenny et al. 2008). Furthermore, conversion of polyethylene pyrolysis wax to PHAs has been documented using *P. aeruginosa* PAO1 (Guzik et al. 2014a).

Apart from the selection of a cheap carbon sources suitable for PHA synthesis, it has to be taken into account that these carbon sources could not be exclusively imposed by local market prices: a global price consistency and the continuous access to these carbon sources have also to be considered. On this basis, the lowering of production costs could be improved if the PHA production plant integrates with industries generating the waste that could be directly accrued as waste streams. Moreover, it must be considered that some of the economical carbon sources to be used as precursors for PHA could increase the costs of their utilization if they should be treated before the fermentation step or if they need to be purified.

21.6.2 Selection of the Optimal Strains

Another aspect to be considered for fine-tuning production costs is the selection of the optimal bacterial PHA producer since a growth-associated PHA producer, those which are able to accumulate PHAs without a growth-limiting compound (e.g., *Alcaligenes latus*, *Paracoccus denitrificans,* or *P. putida* U), would be the choice when using a rich in carbon and nutrients feedstock. However, in these cases when raw material lacks some essential growth nutrient, use of a bacterial strain whose PHA accumulation is not associated with its growth (e.g., *Cupriavidus necator* or *P. putida* KT2440) could be a better choice. Moreover, the strain to be used for PHA production at industrial level must show the metabolic potential to utilize efficiently an inexpensive carbon source and to achieve good rates of growth and polymer synthesis. Furthermore, development of overproducer strains derived from pseudomonads by knocking out genes (e.g., those codifying b-oxidation or some functions of fatty acid de novo synthesis, *phaZ*), augmentation of genes from the same strain (e.g., *phaJ* or *phaG*), expression of modified versions from endogenous genes (e.g., engineered versions of PHA synthase with broader substrate specificity or great processivity), or heterologous expression of convenient genes (e.g., expressing *fadL* from *E. coli*, encoding a fatty acid transporter protein) have been performed. Genetically engineered natural PHA producers are intended to show enhanced substrate conversion, accumulating more PHAs in the cellular biomass and therefore increasing productivity and/or being able to synthesize optimized polyester composition, even producing PHAs containing exotic monomers like scl-monomers or lactate.

Recently, with the increasing knowledge on the genes and enzymes participating in cell morphology, elongasome, biosynthesis of cell wall, and cellular division, divisome, high interest into engineering the morphology of PHA producers has arisen. This interest surged on the basis of increasing the yield of intracellular PHAs wherein increase of cellular volume or induction of filamentous morphology can enlarge the intracellular space for a greater accumulation of polymer and ease the biomass recovery in downstream separation: large cell sizes allow gravity or filtration separation of PHA containing cells and fermentation broth, allowing for a low-cost downstream separation process (Tan et al. 2014; Jiang et al. 2015). However, it has to be considered that interaction with these sensible mechanisms and uncontrolled interference over cell division could lead a drastic reduction in fitness, in cell density, and/or in growth rate of the PHA-producing strains, which could lead to a decrease in the final product yield during fermentation. In such case, an equilibrium between cell growth and morphology modification should be assumed. Moreover, instability of the modified strains should also be considered as mutant or transgenic strains affected in septum formation could show phenotypical changes after continuous subculturing (Humanes, Luengo, Olivera, manuscript in preparation).

However, successful production of PHAs through morphology manipulation has been reported in *P. mendocina* NK-01. In these bacteria, increasing of yield and percentage of mcl-PHA over cell dry weight was obtained through a strategy involving *minCD* deletion and *mreB, ftsZ,* or *sulA* overexpression in the deleted mutant (Zhao et al. 2019).

Systems biology and metabolic engineering have arisen providing new useful tools for planning strategies to increase PHA production. Holistic view of the PHA producer cell provided by systems biology through integration of the datasets from genomics, transcriptomics, proteomics, and metabolomics studies allows for the in silico genome-scale metabolic reconstruction providing a view of the cell as a network showing the interrelationships of pathways and metabolites and allowing for the identification of metabolic bottlenecks. Using these approaches, genome-scale models of *P. putida* KT2440 (*i*JN746, *i*JP815, PpuMBEL1071, *i*JN411) have been proposed (Nogales et al. 2008, 2017b; Puchałka et al. 2008; Sohn et al. 2010). These genome-scale models have been used for studying metabolic features of *P. putida* including the enhanced production of PHAs, reconciliation of key biological parameters for growth on glucose under carbon-limited conditions, and identification of essential genes for growth on minimal medium (Molina-Henares et al. 2010; Poblete-Castro et al. 2013; van Duuren et al. 2013; Borrero-de Acuña et al. 2014; Hintermayer and Weuster-Botz 2017).

As an example of applicability of in silico-driven systems to improve PHA accumulation, on the basis of the analysis of a FluxDesign model, *P. putida* KT2440 was rationally engineered toward enhanced production of mcl-PHAs using glucose as carbon source. The model predicted several beneficial gene deletions, including the gene encoding glucose dehydrogenase, *gcd*. Deletion of this gene have resulted in a 50% increase of the cellular PHA content and an 80% increase in PHA final yield (Poblete-Castro et al. 2013). In addition to the wild-type strains that produce PHAs, or overproducer strains derived from these, genetically recombinant bacteria and plants have been used to produce PHAs from inexpensive carbon sources (Poirier and Brumbley 2010; Dobrogojski et al. 2018; Choi et al. 2020). Thus, genetic engineering and transgenesis aim at various goals: (1) transference of genes encoding PHA synthases, these enzymes plus phasins, or the whole *pha* cluster from a well-known PHA producer, or a combination of different genes coming from different producers, to the appropriate biotechnological chassis, a non-PHA producer, which are easy to culture, mainly in non-expensive media, and (2) directing and optimization of metabolic fluxes through production of PHA precursors in the recombinant organisms containing transgenic PHA biosynthetic genes, through genomic edition of relevant pathways in the chassis, or by introduction of genes needed for the optimal use of a specific carbon source.

E. coli has been used as a workhorse for PHA production using engineered versions of this bacteria affected in β-oxidation and/or fatty acid de novo synthesis. Pioneering studies were reports on PHA accumulation using *E. coli* strains defective in β-oxidative functions after expression of *phaC1* or *phaC2* from *P. aeruginosa* and *P. putida* GPo1 (Langenbach et al. 1997; Klinke et al. 1999; Ren et al. 2000a). From these studies, a plethora of studies have been performed, expressing PHA synthases and/or phasins from different pseudomonads or together with functions for PHA synthesis from other organisms, and studies redirecting metabolic fluxes to PHA accumulation in these recombinant strains (expressing *phaG*, *phaJ*, genes for fatty acid de novo synthesis, or other metabolic functions from exotic organisms) (Leong et al. 2014; Choi et al. 2020).

Industrial use of recombinant organisms could be advantageous as these can produce PHAs without nutrient limitations such as nitrogen or phosphorus, which could lead to a no limited and faster growth and high biomass production which could potentially lead to cost savings. Moreover, transgenic microorganisms are well-known, and their genetic systems (housekeeping and heterologous genes) are clearly defined, allowing for further engineered for metabolic optimization. Bacteria known to be able to utilize certain substrates that the native PHA producers cannot use may also be transformed with PHA-producing genes to produce PHA from potential cheap carbon sources.

21.6.3 Downstream Processing of PHA

Downstream processing also has a great influence over the price of the whole process. In this way, it has been reported that recovery of PHAs after fermentation could reach 30% of the overall production costs (Sun et al. 2007). Nevertheless, availability of amounts of specific PHAs for development of industrial downstream processes is limited, and in many cases, these methods have to be fine-tuned to a specific polymer type (selection of appropriate additives, choice of a specific temperature profile to allow for optimal crystallization rate, etc.) or a specific producer strain. After fermentation, downstream processing involves the microbial mass separation from the culture broth, usually by filtration, flocculation-centrifugation, or centrifugation prior to PHA recovery (Lee 1996b). As PHAs are intracellular polymers, lysis of concentrated cellular mass is currently performed to extract the polymer. Then, PHAs must be extracted from cellular debris with an organic solvent, usually chloroform, dichloromethane, or acetone. These solvents first make the cell membrane more permeable, and then they solve the intracellular PHAs. This step is later continued with a process for PHA concentration and later precipitation with methanol or ethanol (Anderson and Dawes 1990; Ramsay et al. 1994).

However, these retrieval methods are expensive and entails different problems: (1) this method is not appropriate for large-scale purification of PHAs, (2) it has an extra cost for disposal of waste solvents, and (3) it implies the release of an environmental harmful waste, which represents a contradiction with consideration of PHA as environmentally friendly polymers. With regard to this, environmentally friendly solvent systems are being investigated (Fei et al. 2016; Aramvash et al. 2018). On the other hand, this kind of extraction allows for the obtaining of highly purified PHAs with negligible degradation of polymer (Ramsay et al. 1994; Kunasundari and Sudesh 2011). Nevertheless, some researchers have evaluated alternative nonsolvent-based techniques for PHA recovery. Mechanical cell-disruption methods such as using bead mills or high-pressure homogenization are effective and inexpensive (Jacquel et al. 2008). Bead mill mechanical treatment has, as major drawback, the long time needed for processing and the number of parameters that should be optimized (Kunasundari and Sudesh 2011). On the other hand, the high pressure in homogenization process could affect the polymer promoting its thermal degradation (Diels and Michiels 2006). Other methods imply chemical treatment of the cellular mass for digestion of non-PHA cell mass. These methods could be classified as chemical or enzymatic digestion. Chemical digestion of non-PHA cellular material also has disadvantages. For example, hypochlorite can degrade the polymers (Ryu et al. 2000; Heinrich et al. 2012) although enzymatic degradation (Kapritchkoff et al. 2006; Israni et al. 2018) is too expensive to be economically reasonable.

Additional methods have been proposed, as supercritical fluid disruption (Hejazi et al. 2003; Khosravi-Darani et al. 2004), combined treatment with ethylenediaminetetraacetic acid and microwave cell lysis (Balakrishna Pillai et al. 2018) or dissolved air flotation (van Hee et al. 2006); however, these techniques still need to be further developed. A different approach relies on the use of genetic engineered circuits triggering off autolysis of producer cells. Under this premise, induced autolysis, strains in which induction of holin-endolysin phage lysis system (Liu and Curtiss 2009; Martínez et al. 2011; Tamekou Lacmata et al. 2017), heterologous expression of lysozyme to enable degradation of the peptidoglycan layer (Borrero de Acuña et al. 2017), and overproduction of OprE and OprF assisting cell lysis under severe osmotic stress (Poblete-Castro et al. 2020a) have been developed. However, these autolytic mechanisms still face several technical limitations. Finally, as a novel method for PHA recovery, the use of animals (mealworms or rats) fed with dried bacterial cells containing PHA with recovery of polymers from their feces has been also proposed (Murugan et al. 2016; Ong et al. 2018a, b).

21.7 Conclusion

PHAs represent a promising group of biodegradable plastic polymers with several advantages over petrochemical plastics that will allow their replacement in a close future. Research on mcl-PHAs produced by pseudomonads in the last 40 years and an integrative view of omic technologies are allowing the development of new strains and culture conditions that will permit the increase of production and the tailoring of interesting polymers. Thus, flux optimization for metabolic targeting in the production of specific monomeric precursors that will be polymerized on specific rationale-tailoring plastics is one of the research topics for empowerment of the commercial production of these bacterial plastic polymers. However, more research will be necessary to understand the intricacies of carbon metabolism in these strains and to provide new target genes to develop better PHA producer strains. Moreover, fermentative improvements will also allow for the obtaining of new polymers with exotic mechanical and physicochemical properties and, consequently, new applications in consumer goods that would be demanded by society. Furthermore, the possibility of blending with other polymers and the chemical modification of the biological polymers will open the range of this new applicability.

On the other hand, the wide metabolic versatility of pseudomonads would also imply the cheapening on PHA production. The use of wastes derived from other economic processes would lead to the obtaining of PHAs at a competitive cost. Unfortunately, due to the difference in today's production costs, between plastics of petrochemical origin and PHAs, investor's interest in product development has, until now, been limited though it is expected that, in a short time, there will be an application window in which PHAs can compete with other materials in specialized markets. In addition, the social demand for the development of a sustainable green lifestyle must be considered. As the human being becomes more and more aware of environmental abuse, climate change, and the associated risks to our health, the demand for healthier and more suitable alternatives is becoming a priority. Therefore, our duty as scientists is to develop the different aspects needed for this change, promoting the development of the technologies necessary for the introduction of PHAs as one of the best alternatives to nonbiodegradable plastics. This paradigm shift will depend on the fine-tuning of a whole pool of knowledge that has been developed in research laboratories and that must be scaled up for industrial implementation.

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Application of Bioplastics in Agro-Based Industries and Bioremediation

22

Sharjeel Ahmad, Saira Abbas, Nauman Khalid, Ahmad Ali, and Iftikhar Ahmed

Abstract

Bioplastics are the plastics composed from the biomass of corn, sugarcane, etc., which nowadays form an eco-friendly system that saves fuel burning, CO_2 emission, and plastic. There are emerging interests in agro-based research packaging for designing cost-effective bioplastics, thanks to the heavy burden of diseases from petroleum products. Plastic industries are among the foremost important and regularly applied industries within the globe, and they are widely employed due to its biodegradability nature. Polyhydroxybutyrate (PHB) and polyhydroxyalkanoate (PHA) are naturally produced by microorganisms that can be considered as a substitute for the standard plastics. These biodegradable materials are currently providing a sustainable alternative to petroleum feedstock. Mostly, biodegradable polymers get hydrolyzed into biomass, CH_4, CO_2, and other inorganic compounds. The utilization of bioorigin materials in food packaging has led to the tremendous innovation during last couple of years, which were obtained through microbial fermentations (e.g., starch and cellulose).

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The plant-based bioplastics are considered to be more durable, cost-effective, chemical independent, highly immune to microwaves, and less brittle. It is concluded that the assembly and use of bioplastics will aid in the sustainability of the environment by reducing greenhouse gases (GHGs), reduction in waste biomass, and as a substitute to petroplastics.

Keywords

Bioplastics · Pros and cons of bioplastics · Applications · Production of bioplastics

22.1 Introduction

Bioplastics can be defined as the plastics based on renewable resources mainly from plant-based starch like cornstarch, potato starch, and fibers like banana stem, henequen leaves, jute, hemp, and pineapple; these bioplastics are considered as bio-based or biodegradable and/or compostable plastics (Sudesh and Iwata 2008; Siracusa et al. 2008). Conventional plastics not only become toxic during degradation but also take an extended period for decay (Gross and Kalra 2002). These plastics are made of non-degradable sources like petroleum material, etc., which are currently used in different agro-based industries (Pavia et al. 1988). These sorts of materials are made by a carbon-carbon linkage which makes them difficult to degrade (Rader and Stockel 1995). Therefore, there is a requirement of the materials which are degradable, recyclable, and eco-friendly (Gross and Kalra 2002). Bioplastics are the naturally synthesized biopolymers which are catabolized by microorganisms and accumulated into their body cells under stress condition as a storage material (Berlanga et al. 2006; Kadouri et al. 2005; Bharti and Swetha 2016). Different biodegradable polymers (Fig. 22.1) are utilized in various fields (Saharan and Ankita 2012); for example, in the production of poly-*p*-dioxanone (PPDO), polybutylene succinate (PBS), polylactide (PLA), poly-*ε*-caprolactone (PCL), including polyhydroxyalkanoates (PHAs) (Witholt and Kessler 1999). Bioplastics are often made by utilizing microorganisms and a few nanoparticles like carbohydrate chains (polysaccharides) (Jamshidian et al. 2010; Petersen et al. 1999; Zepnik 2010; Sorrentino et al. 2007). Due to awareness of the environmental safety concerns among people, the focus is shifted more towards the development of biodegradable materials and away from petroleum-based plastics (Kalia et al. 2000a; Saharan and Ankita 2012). A new policy on bioeconomy is developed by the Organization for Economic Cooperation and Development (OECD) by linking renewable biological and bioprocess resources through high-put biotechnologies for generating incomes and jobs (Kerry and Butler 2008; Primer 2001). Moreover, new biodegradable food packages are designed for increasing safety and shelf life of fruits and vegetables (Dallyn and Shorten 1988). Biodegradable packaging industries are booming due to increased consumer preferences, because of better quality, safety, and environment friendliness. Consequently, these technologies have lead to research in the field of

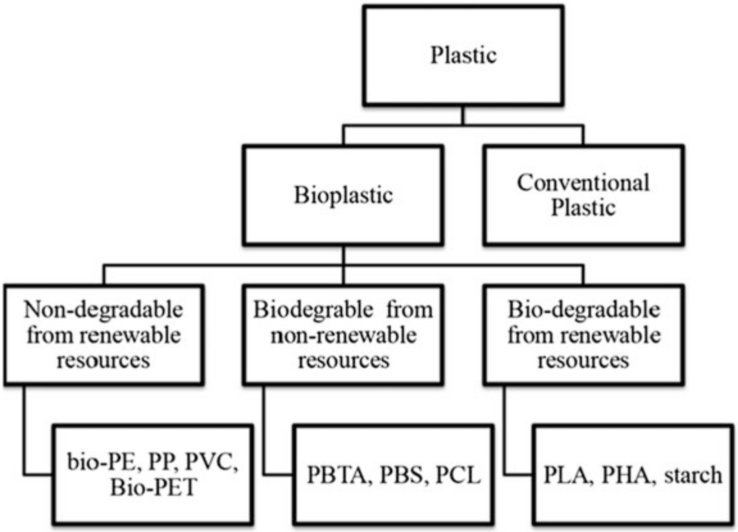

Fig. 22.1 Classification of plastics

bioplastics and biopolymers originating from marine and agricultural sources (Lopez-Rubio et al. 2004; Comstock et al. 2004; Cha and Chinnan 2004). Bioremediation is a biological and organic process for transforming contaminants into useful products using microbiological associations (Panigrahi and Konar 1992). If primary goal is controlling GHG emissions with increasing the shelf life of plastics and its recycling, then the production pattern will shift towards bio-based plastics which will likely to be acceptable in future (Kerry and Butler 2008). The Intergovernmental Panel on Climate Change (IPCC) tries to scale back atmospheric greenhouse gas (GHG) emission about 80% to 2050 as compared to 1990 by minimizing carbon dioxide (CO_2) concentration (Metz et al. 2007). This chapter covers the bioplastics classification, production, advantages, disadvantages, and its applications in agro-based industries and bioremediation.

22.2 Classification of Bioplastics

The characteristics of all types of bioplastics are shown in Table 22.1.

22.2.1 Types of Bioplastics According to Jabeen et al. (2015)

22.2.1.1 Microbe-Based Bioplastics

Microorganisms through the biodegradation process disintegrate the natural polymers and synthesize the polymer (synthetic polymers which are known as bioplastics) (Koller and Owen 1996). This degradation process includes the erosion,

Table 22.1 Materials for bioplastics and its characteristics

Materials	Characteristics
Cellulose acetate (CA)	• Cellulose is formed from wood pulp or linters of cotton • This is not 100% pure cellulose • Good chemical resistivity • Moderate ability to circumvent friction • Good elongation at break • Protective against insects • Poor abrasive ability • Poor breaking tenacity • Absorbs water easily, loses strength when wet • Soluble in acetone • Weak against strong bleaching/oxidizing agents • Soluble in acids • Damages greatly by strong alkalis • Relieves dyes easily, needs special dyes and pigments to color it • Easy to bond with plasticizers, heat, and pressure • Can be incinerated or composted • Cellulose acetate is tough, deeply glossy, smooth, and transparent material and feels quite natural. Due to its many beneficial characteristics, it is widely used for the production of many general products
Polyamide (PA)	• Exhibits lower water absorption • Has lower brittle temperature • Has good abrasion resistance and chemical resistance • Possesses lower strength and stiffness • Low coefficient of friction • Good electrical insulating properties • High resistance against high energy radiation (gamma and X-rays)
Polyethylene (PE)	• Economical • Low coefficient of friction • Excellent chemical resistance • Stable in cryogenic environments • Good impact resistance • FDA/USDA approved (HDPE) • Resistant to many solvents (HDPE) • Good fatigue and wear resistance (HDPE) • Zero water absorption (HDPE)
Polylactic acid (PLA)	• Production uses 30–50% less fossil energy • Generates 50–70% less CO_2 emissions than PBS (Polybutylene succinate) • Competitive use of water with the best performing PBS • Recyclable • Compostable at temperatures above 60 °C
Thermoplastic starch (TPS)	• Production requires 68% less energy than its PBS • Lower CO_2 emissions than PBS • Biodegradable • Compostable

dislocation, phase separation, and cracking of polymers like polythene which acts as a substrate for microbes (Mukherjee and Chatterjee 2014). Plastics are being biodegraded by reducing the polymer chain length which oxidizes the polymers; then the changes are assessed by a rapid rise in microbial growth under biotic conditions and by CO_2 production (Bhardwaj et al. 2013). The flowchart of this type of bioplastic is shown in Fig. 22.1.

Starch-Based Bioplastics

Starch is utilized as an initial material for the extensive range of green materials because it is 75% of all organic materials on earth (an important form of polysaccharides). It is synthesized and stored within the plant structure as their energy source and therefore found in seeds and in tubers or roots of the plants (Asaf Kleopas 2008). Starch constitutes of amylose and amylopectin. Amylose comprises of 500 to 20,000 D-glucose units mainly linked with α(1–4) glycosidic bonds, while amylopectin is composed on up to 30 branched α(1–6) glycosidic bonds attached to primary α(1–4) glycosidic bonds. The amount of amylose and amylopectin depends on the type of plant resources like wheat, pea, tapioca, rice, corn, potato, etc. (Wool and Sun 2005; Ali et al. 2017; Huda et al. 2005, 2008; Hu and Lim 2007; Niranjana Prabhu and Prashantha 2018; Wang et al. 2008; Zhao et al. 2008; Graupner 2008; Avella et al. 2008; Tokoro et al. 2008; Shikamoto et al. 2009). The production of starch-based bioplastics is shown in Fig. 22.2. According to Gadhave et al. (2018), there are five kinds of starch-based bioplastics which are utilized in the market including compostable polymers, nanocomposite films, anti-microbial packaging films, starch grafting films, and heat sealing packaging. Starch-based bioplastics have flexible films which contain polyesters to enhance processing ability, water resistance, and tear strength (Marvizadeh et al. 2017).

22.2.1.2 Chemically Synthesized Bioplastics

Polycaprolactone and polyvinyl alcohol (PVOH) are synthetically prepared from petroleum resources, although its biodegradability is disputed based on water solubility. The water-soluble polymer PVOH, which is widely employed, is made by the hydrolysis of polyvinyl acetate (Kumar and Thakur 2017). Different bioplastic properties like heat stability, thermal instability, high water vapor, oxygen permeability, and brittleness affect the usage in food applications (Jong-Whan et al. 2009). Green chemistry is meant to assist us to aim not just to treat waste but to avoid producing such wastes which are hazardous for health (Höfer and Selig 2012; Anastas and Warner 1998; Ward and Wyllie 2019). According to Anastas and Warner (1998), the basic principle of green chemistry is to prevent chemically synthesized wastes, design of safe chemical products which are fully effective,

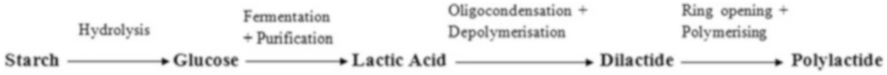

Fig. 22.2 Starch-based plastic production

design of the less hazardous chemically synthesized wastes with little or no toxicity, use of renewable feedstock, use of eco-friendly catalysts, minimum use of chemical derivatives, and eco-friendly solvents (Ward and Wyllie 2019; Fisher et al. 2007).

22.2.2 Categories of Bioplastics According to Musiol et al. (2016)

22.2.2.1 Nonbiodegradable Bioplastics Derived from Renewable Resources

Bio-Polyethylene (PE)

It is also called organic polyethylene (PE) synthesized using raw materials from agricultural feedstocks like corn, sugarcane, etc. (Morschbacker 2009). As of plastic production worldwide, PE is the dominant polymer which is of various types based on the different manufacturing conditions and properties which may alter due to attached additional hydrocarbons like 1-propane, 1-pentane, 1-hexane, 1-heptane, and 1-octane (Koopmans 2014).

Polypropylene (PP)

The second most commonly used plastic is polypropylene which is originated from renewable feedstock sources (Harracksingh 2012). Propylene can be available from methane by ethanol dimerization through metathesis (Popoff et al. 2013; Rechnia et al. 2012). Bio-based PP became popular due to high demand in automotive industries as about 50% of the plastic of the car contains PP (Greene 2014).

Polyethylene Terephthalate (PET)

PET is a partial bio-based polyester that can be produced from fossil-based terephthalic acid and bio-based 1,3-propanediol (1,3-PDO) or ethylene glycol (Zeng and Sabra 2011). According to Potera (1997), these bio-based materials are formed by the direct fermentation of recombinant *Escherichia coli* bacteria and glucose (substrate). Due to rapid advancement in gene-splicing, different genes of bacteria are combined such as for the production of glycerol from 1,3-PDO by the combination of genes of *Klebsiella pneumonia* and *E. coli* (Zeng and Sabra 2011).

22.2.2.2 Biodegradable Bioplastics Derived from Nonrenewable Resources

Polybutylene Adipate-Co-Terephthalate (PBTA)

It is an aliphatic-aromatic biodegradable co-polyester which is derived from acids (terephthalic acid and adipic acid) together with butanediol using polycondensation reaction resulting in good strength and the processing ability that is widely used for creating wrapping films, mulch films, disposable tableware, and packaging materials and different organic waste bags (Havstad 2020; Muthuraj 2015).

Polybutylene Succinate (PBS)

It is a biodegradable aliphatic thermoplastic polyester which is formed by the condensation of the polymerization process of succinic acid and 1,4-butanediol with the assistance of a catalyst. However, PBS is mainly derived from fossil-based monomers and can also be obtained as a result from bio-based monomers or a combination of bio-based and fossil-based monomers. PBS is employed for several applications which include shopping bags, plant pots, packaging films, and many agricultural mulch films (Havstad 2020; Barletta et al. 2020).

Poly-e-Caprolactone (PCL)

PCL is a biodegradable polymer having low melting point around 60 °C and is most used plastics. Its properties make it favorable to use in biomedical field like surgical suture and for rapid prototyping (immersion in hot water causing softening) (Liu et al. 2010; Ebnesajjad 2012).

22.2.2.3 Biodegradable Bioplastics Derived from Renewable Resources

Polylactide (PLA)

Polylactic acids (PLAs) are easily compostable and injection-molded bioplastics (Starr and Westhoff 2000), that makes this compound alternative to petroleum-based polymers for the synthesis of single-use food packaging items. PLA also produces less than 70% greenhouse gases during landfill degradation (Stevens 2013). Lactic acid (formed as a result of algae fermentation) is also another raw material for the synthesis of bioplastics. The polymerization process of lactic acid yielded polylactic acid (PLA), which is also biodegradable version and economically viable in comparison to conventional plastic (Brehmer 2014; Farah et al. 2016). The production of PLA is shown in Fig. 22.2.

Polyhydroxyalkanoates (PHAs)

PHAs are mainly comprised of 3-hydroxyalkanoates as a monomeric building block (Šprajcar et al. 2012) and contain a family of polyesters formed as the intracellular products by numerous prokaryotic genera which include Gram-positive and Gram-negative eubacteria, cyanobacteria, and even archaea under extreme environments (Abreu et al. 2017; Koller and Owen 1996). According to Ehrenhauser (2015), PHAs (also known as poly-4-alkan-2-oxelanones) have become alternative polymeric materials that originated from natural renewable and biowaste materials (Volova 2004a, b).

Polyvinyl Alcohol (PVA)

It is a water-soluble and biodegradable polymer, which possesses biocompatibility and has capacity of self-cross-linking due to the presence of higher amount of hydroxyl groups on its side chains (Guo et al. 2013). PVA can undergo hydrolysis and as a result becomes bio-inert. Its application includes water treatment channels, agricultural chemicals, dyes, etc. (McKeen and Massey 2017; Trapani 2014; Havstad 2020).

Bioplastics Based on Polysaccharides

Polysaccharides are the most abundantly found macromolecules within the biosphere having monosaccharides bound together by glycosidic bonds and are the complex carbohydrates which are found in the form of starch (as it plays a key role in plant energy storage), cellulose, chitin, and pectin (Chivrac et al. 2009).

Starch in Granular Form

Starch granules are of different shapes (spherical, polygonal, etc.) and of different sizes (0.5–175 μm), depending upon their biological origin (Guilbot and Mercier 1985). The starch types are shown in Fig. 22.3. The starch granules have alterations in different amorphous and crystalline surfaces based on amylose and amylopectin contents. The crystalline surfaces contain amylose chains as their side chains, while amorphous areas are constituted with both amylose and amylopectin chains (Jenkins 1995; Van Soest et al. 1996; Chivrac et al. 2009). The native starch is biologically synthesized and assembled in the form of semicrystalline granules which are labeled as A, B, or C type depending upon the origin of starch (Chinnaswamy et al. 1989; Eliasson 1994; Riisom et al. 1984).

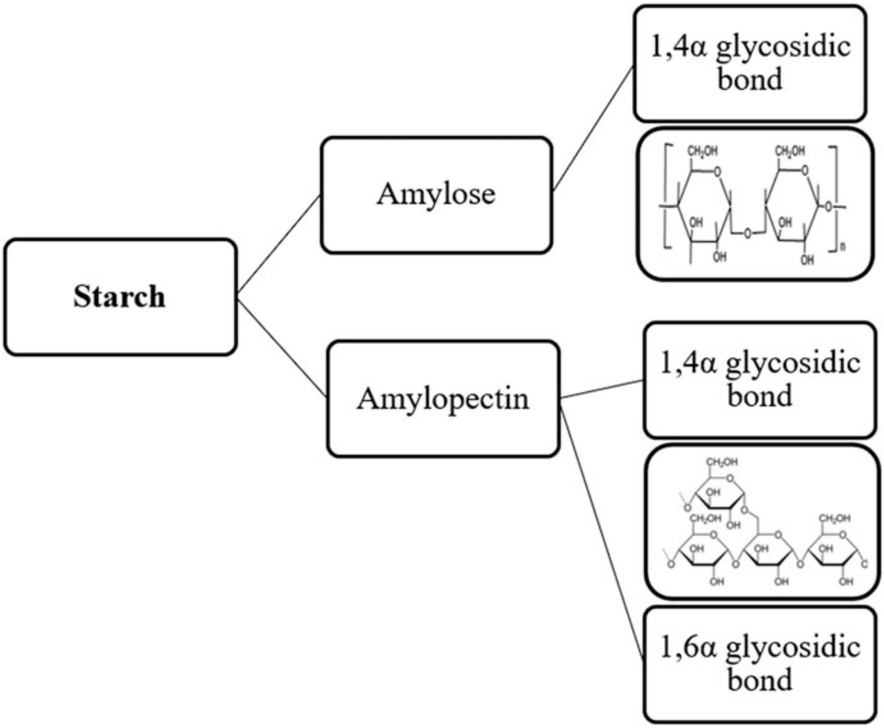

Fig. 22.3 Starch composition and its structure bonds

Gelatinized Starch

Starch granule structure can be gelatinized by melting the polymer to proper mixing of two different polymers together under controlled moisture condition (Albertsson and Karlsson 1995). Many researchers focused on starch found from corn, wheat, potatoes, etc., but also a number of them reported sago starch which is made from sago palm (*Metroxylon* sp.) (Mohamed et al. 2008). Sago starches, also known as rumbia, are found from Southeast Asia (Sarawak and Malaysia). Sago starch is employed as the ingredient in food industries as a gelling agent (Zuraida et al. 2012). It requires the combination of other polymers with it to enhance its properties like starch modification (melting intrusion of thermoplastic starch (TPS), grafting, and esterification) (Carvalho 2008). The modified starch is named as bioplastic starch (BPS), which is an amorphous or semicrystalline polymer comprised of gelatinized starch that have a mixture of one or more plasticizers and additives (Liu et al. 2009; Shi et al. 2007; Carvalho 2008).

Thermoplastic Starch (TPS)

Thermoplastic starches are prepared by the rapid breakdown of starch granules in the existence of plasticizers or additives under low moisture content and high temperature and pressure (Tomka 1991; Swanson et al. 1993). Environmentally friendly fillers are used to improve the properties of TPS as it cannot meet the requirements of the packaging materials. Clay is one of the best potential fillers, which is naturally found abundant mineral that has non-toxic property and is also one of the most important components of food chain (Bakraji and Karajou 2003; Jinadasa and Dissanayake 1992; Pang et al. 2003; Qian et al. 2002). TPS has very hydrophilic properties and processes just like the conventional plastics (Averous et al. 2000; Chen and Evans 2005). Some researches included efforts to improve starch structure by reducing hydrophilic character with the addition of acetylation by which it leads to lower mechanical properties and high cost (Fringant et al. 1996). Some authors also explained the alteration in the mechanical properties in relation to the crystallinity of TPS during the aging process which leads to the association of TPS with other biopolymers for restoring the biodegradability of the final material (Van Soest et al. 1996; Van Soest and Knooren 1997; Koenig and Huang 1994). These associations are in the form of blends or multilayered products (by coating or by co-extrusive processes) (Avérous et al. 1999; Koenig and Huang 1994). Blended TPS are nowadays commonly employed in different fields (Chiellini and Solaro 1996; Amass et al. 1998; Jasberg et al. 1992; Bastioli 2002; Lörcks 1998).

Modified Starch

The starch molecule is hydrophobic in nature. As regarding the bioplastic preparation, different properties like thermal stability and strength need to be improved. This can be achieved by modification of its properties; for example, addition of ester groups through blending mechanism can improve thermal stability, moisture absorption, water vapor transmission rate, and barrier transfer for different gases (Albertsson and Karlsson 1995; Lim et al. 1992; Ellis et al. 1998).

22.3 Advantages and Disadvantages of Bioplastics

The pros and cons of bioplastics are shown in Table 22.2.

22.3.1 Advantages

Reducing CO_2 emission: According to Reddy et al. (2013), one metric ton of petroleum-based plastics produces 0.8–3.2 metric tons of carbon dioxide as compared to same quantity of bioplastic.

Waste management: The best advantage of bioplastics is its biodegradability which helps in waste management and permanent litter reduction (Shamsuddin et al. 2017).

Providing long shelf life: According to Woodford (2008), it provides long shelf life to food.

Eco-friendly: Bioplastics generate less amount of greenhouse gases (GHGs) and contain no toxins (Arikan and Ozsoy 2015). Its biodegradable nature avoids the danger of pollution and provides easy-to-use accessibility.

Independence: It is made from renewable resources such as corn, sugarcane, etc., as compared to petroleum-based plastics (Yu and Chen 2008).

Energy efficiency: Its production uses less amount of energy. In contrast, the plastics are composed from about 4% of the oil that the world uses every year. With the scarcity of oil, the manufactures of plastics are increasingly exposed to cost fluctuations (Reddy et al. 2013; Arikan and Ozsoy 2015; Chen 2014; Singh and Devi 2019).

Lower carbon footprint: Since bioplastics are produced from renewable resources that capture CO_2 using photosynthesis, this sequestration process recycles CO_2 and results in environmental protection, but synthetic polyethylene or other conventional plastics store CO_2 forever (Shamsuddin et al. 2017; Reddy et al. 2013; Chen 2014; Arikan and Ozsoy 2015).

Table 22.2 Pros and cons of bioplastics

Advantages	Disadvantages
Lower fossil fuel consumption	Compostability
Less dependency on nonrenewable resources	Made of plant biomass, therefore causes contamination
Lower CO_2 and other GHG emissions	Nonrecyclable
Decrease in waste generation	High cost
Water saving	Need large area
Potentially much lower carbon footprint	Engineering problem
Energy efficiency	GMOs are employed for its manufacturing
Independence	Contaminate plastic recycling streams

22.3.2 Disadvantages of Bioplastics

Food crisis/land required for renewable resources: As the population is growing rapidly, the food requirement for feeding population masses is also increasing rapidly. There is almost one million tons of bioplastics being produced annually, which can be employed for renewable food reservoir. If bioplastic production and demand continued to increase rapidly, a large area of land is expected to be taken from the food stock for its production instead of human dietary needs (Ploetz et al. 2016).

Compostability: Taiwan is the only country with industrial composters, but other countries lack such kind of facilities, which means all the food packed in bioplastic containers coming at the supermarket ends up in the landfills (Emadian et al. 2017).

Recycling of bioplastics could become impossible: The bioplastics are biodegradable but not recyclable, however, bioplastics can damage the recycling process and blocks drains (Cyras et al. 2007).

Limited area for bioplastics degradation: Most developed countries in the world have recycling workshops but rarely have industrial composters. Therefore, proper management for its disposal is not yet settled. This can create different detrimental consequences on environment (Rhim et al. 2009).

Engineering problems: Biodegradable plastics are mainly composed of plant biomasses such as soybeans and corn. At the time of their growth period, different pesticides, herbicides, or fungicides are used which can cause contamination in the food products (Modi 2010).

Requirement of expensive equipment: A major drawback of bioplastics is that there is a need of commercial processors and composters for both of its manufacturing and disposal which make it costly (Jong-Whan et al. 2009).

Bioplastics contaminate plastic recycling streams: As mentioned above, bioplastics can contaminate recycling materials and make it unstable, brittle, and useless, which become non-recyclable (Chidambarampadmavathy et al. 2017).

22.4 Processing of Biopolymers

22.4.1 Production of Bio-Based Polymers from Renewable Wastes and Agro-Wastes

22.4.1.1 Thermoplastic Starch-Based Polymers

Materials: Starch is usually taken from corn products because of its amylose content (28%). Natural rubber latex can also be used which is directly extracted from rubber tree (*Hevea brasiliensis*). Its solid contents were measured by drying the emulsion in an oven for about 12 h at 110 °C which is almost 35 wt%. Usually glycerol is used as a reagent in the commercial production of TPS (National Research C 1994; Carvalho et al. 2003). In the United States, the thermoplastics produced are of different kinds whose production percentage is given in Table 22.3.

Table 22.3 Diversity of thermoplastic production by the United States

Thermoplastic type	Production (%)
Polyvinyl chloride	19
Polypropylene	18
High-density polyethylene	18
Low-density polyethylene	15
Polystyrene	11
Liner low-density polyethylene	8
Thermoplastic polyester	4
Acrylonitrile butadiene styrene	3
All others	4

Preparation: The glycerol-to-starch proportion is maintained which varies from 20% to 50% in the blend formation. The dry rubber content can be varying from 2.5% to 20% to the TPS with respect to total dry weight. Hand mixing of starch, glycerol, and latex was done to obtain a homogenized mixture in the beaker. Haake Rheomix 600 intensive batch mixer equipped with roller rotors is employed for proper mixing at 150 °C and 50 rpm for 6 min. At the end, this material is hot-pressed at 150 to 160 °C. This aids in the conversion of material into plates of different sizes and shapes (Willett et al. 1994).

Mechanical tests: Usually a testing machine (Instron, model 5500-R, operated at 550 mm/min) is employed for stress-strain tests of plates, which were cut into dumbbell shapes. This will help in shaping of material in accordance with the ASTM D 638 M standard. The above-mentioned machine can also be employed to measure the strain at break (ε_r), which is the ultimate tensile strength (UTS), and the secant elastic module at 1% strain ($E_{1\%}$). It is basically a 4-week conditioning of each material to be tested at 25 °C temperature and 53% relative humidity (Curvelo et al. 2001).

Scanning electron microscope (SEM): The above blended mixture obtained is cooled in liquid nitrogen and studied by a Digital Scanning Electron Microscope (model DSM 960 Zeiss, operated at 10–15 kV), equipped with tungsten filaments, further coated with the layer of gold (Greve 1993; Carvalho et al. 2003).

22.4.1.2 Production of Polylactic Acid (PLA) from Agro-Wastes

There are two methods used to manufacture PLA.

Polymerization

The first method is the production of PLA by direct polymerization of lactic acid which results in the formation of low molecular weight polymers which is done by the production of lactide, in the lactic acid cycle, which also acts as an intermediate source for the formation of PLA with high relative molecular weight.

Condensation polymerization: It is one of the simplest methods to produce PLA. Lactic acid monomers, which contain hydroxyl and carboxyl groups, under the action of self-esterification produce the reversible step growth polymerization. Water is produced as a by-product in this method which is then removed by high temperature and vacuum for avoiding the reversibility of the reaction. The presence of water results in the formation of lower relative molecular weight polymers. This is a costly process as it needs rector, temperature management, and vacuum condition but produces low-quality product which is the main drawback of this method (Maharana et al. 2009; Vink et al. 2003).

Ring-opening polymerization: It is an easy and common method for attaining high molecular weight PLA. The PLA produced from the intermediate lactic acid polycondensation that is from the lactide (it is the lactic acid cyclic dimer produced during dehydration of lactic acid oligomers). It is possible that different lactides are obtained as the lactic acid is the most active compound. Different lactides are formed as a result of the mixture of enantiomers, which act as a substrate of the reaction. Three stereoforms are obtained by the combination of D-lactic acid and L-lactic acid which are D-lactide, L-lactide, and meso-lactide (Board 2006; Lopes et al. 2014; Coudane et al. 1997; Vert et al. 1995).

Melt polycondensation: Some researchers like Masutani and Kimura (2014), Song and Wu (2011), Fukushima et al. (2000), and Moon et al. (2001) have explained the concept of melt polycondensation for the first time. In this method, the dehydration of L-lactic acid (LLA) (it is an organic acid whose formula is $C_3H_6O_3$) solution is carried to get rid of water contents, and then oligomerization is done to obtain LLA oligomers. The LLA oligomers are then polycondensed to get PLA prepolymer, which is crystallized during the solid-state polycondensation (SSP) to obtain high molecular weight PLA in solid state under melting condition (Hangzhen 2001; Hangzhen and Bing 2002; Kucharczyk et al. 2011).

Fermentation

There are two types of fermentation processes used which aid in the production of PLA that is the fermentation of lactic acid and of bacterial polyester fermentation. Fermentation of lactic acid is the conversion of produced lactic acid into polylactic acid by using traditional polymerization processes. Bacterial polyester fermentation is the process of employing bacteria to produce PLA which is yielded as a by-product when converting sugar of corn plants into polyester.

22.4.1.3 Production of Polyhydroxyalkanoates (PHAs) from Agro-Wastes

Microorganisms: There are numerous microbes which are being employed for the production of PHA. Some important bacterial strains are *Bacillus* sp., *Pseudomonas* sp., *Alcaligenes* sp., *Escherichia coli*, *Halomonas boliviensis*, etc., but the most important one is *Cupriavidus necator* which is recently broadly studied (Vandamme and Coenye 2004). A numerous number of microbial strains used for the production of PHA are shown in Table 22.4.

Media: The choice of media depends upon different factors like microbes need, strain type, cost-effectiveness, and production of homo- or co-polymer (Ojumu et al. 2004; Saharan et al. 2007; Serafim et al. 2008; Mohan and Reddy 2013). But generally the media chosen are comprised of different ingredients which are activated sludge, swine waste, starch and starchy wastewater, whey, molasses, effluents from olive mills and palm oil mills, corn steep liquor, and wheat and rice

Table 22.4 Microorganisms employed for the production of PHA (Leung 2004; Singh Saharan et al. 2014; Getachew and Woldesenbet 2016; Mostafa et al. 2020; Kumar and Thakur 2017)

S. no	Microbial strains	S. no.	Microbial strains
1.	*Acidovorax* sp.	**2.**	*Leptothrix* sp.
3.	*Acinetobacter* sp.	**4.**	*Methanomonas* sp.
5.	*Actinobacillus* sp.	**6.**	*Methylobacterium* sp.
7.	*Actinomycetes* sp.	**8.**	*Methylocystis* sp.
9.	*Aeromonas* sp.	**10.**	*Methylomonas* sp.
11.	*Alcaligenes* sp.	**12.**	*Methylosinus* sp.
13.	*Allochromatium* sp.	**14.**	*Methylovibrio* sp.
15.	*Anabaena* sp.	**16.**	*Micrococcus* sp.
17.	*Aphanothece* sp.	**18.**	*Microcoleus* sp.
19.	*Aquaspirillum* sp.	**20.**	*Microcystis* sp.
21.	*Asticcacaulis* sp.	**22.**	*Microlunatus* sp.
23.	*Azomonas* sp.	**24.**	*Moraxella* sp.
25.	*Azospirillum* sp.	**26.**	*Mycoplana* sp.
27.	*Azotobacter* sp.	**28.**	*Nitrobacter* sp.
29.	*Bacillus* sp.	**30.**	*Nitrococcus* sp.
31.	*Beggiatoa* sp.	**32.**	*Nocardia* sp.
33.	*Beijerinckia* sp.	**34.**	*Nostoc* sp.
35.	*Beneckea* sp.	**36.**	*Oceanospirillum* sp.
37.	*Brachymonas* sp.	**38.**	*Oscillatoria* sp.
39.	*Bradyrhizobium* sp.	**40.**	*Paracoccus* sp.
41.	*Burkholderia* sp.	**42.**	*Paucispirillum* sp.
43.	*Caryophanon* sp.	**44.**	*Pedomicrobium* sp.
45.	*Caulobacter* sp.	**46.**	*Photobacterium* sp.
47.	*Chloroflexus* sp.	**48.**	*Protomonas* sp.
49.	*Chlorogloea* sp.	**50.**	*Pseudomonas* sp.
51.	*Chromatium* sp.	**52.**	*Ralstonia* sp.
53.	*Chromobacterium* sp.	**54.**	*Rhizobium* sp.
55.	*Clostridium* sp.	**56.**	*Rhodobacter* sp.
57.	*Comamonas* sp.	**58.**	*Rhodococcus* sp.
59.	*Corynebacterium* sp.	**60.**	*Rhodopseudomonas* sp.
61.	*Cupriavidus* sp.	**62.**	*Rhodospirillum* sp.
63.	*Cyanobacterium* sp.	**64.**	*Rubrivivax* sp.
65.	*Defluviicoccus* sp.	**66.**	*Saccharophagus* sp.
67.	*Delftia* sp.	**68.**	*Shinorhizobium* sp.
69.	*Derxia* sp.	**70.**	*Sphaerotilus* sp.
71.	*Ectothiorhodospira* sp.	**72.**	*Spirillum* sp.
73.	*Erwinia* sp.	**74.**	*Spirulina* sp.
75.	*Escherichia* sp.	**76.**	*Staphylococcus* sp.
77.	*Ferrobacillus* sp.	**78.**	*Stella* sp.
79.	*Gamphospheria* sp.	**80.**	*Streptomyces* sp.
81.	*Gloeocapsa* sp.	**82.**	*Synechococcus* sp.
83.	*Gloeothece* sp.	**84.**	*Syntrophomonas* sp.

(continued)

Table 22.4 (continued)

S. no	Microbial strains	S. no.	Microbial strains
85.	*Haemophilus* sp.	**86.**	*Thiobacillus* sp.
87.	*Haloarcula* sp.	**88.**	*Thiococcus* sp.
89.	*Halobacterium* sp.	**90.**	*Thiocystis* sp.
91.	*Haloferax* sp.	**92.**	*Thiodictyon* sp.
93.	*Halomonas* sp.	**94.**	*Thiopedia* sp.
95.	*Haloquadratum* sp.	**96.**	*Thiosphaera* sp.
97.	*Haloterrigena* sp.	**98.**	*Variovorax* sp.
99.	*Hydrogenophaga* sp.	**100.**	*Vibrio* sp.
101.	*Hyphomicrobium* sp.	**102.**	*Wautersia* sp.
103.	*Klebsiella* sp.	**104.**	*Xanthobacter* sp.
105.	*Lamprocystis* sp.	**106.**	*Zoogloea* sp.
107.	*Lampropedia* sp.		

bran (Halami 2008; Bhubalan et al. 2007; Sim et al. 2001; Saharan and Ankita 2012; Tamis et al. 2014; Nikel et al. 2006; Solaiman et al. 2006; López et al. 2015). The composition of media used for growing these microorganisms are given in Table 22.5.

Fermentation process: There are many investigations done on the fermentation process for the production of PHA to find out what kind of fermentation is best for it (Choi and Lee 1999; Durner et al. 2010; Kim et al. 2007; Zinn et al. 2001). The fermentation process is largely counting on the numbers of microbial cultures, which are being employed for the formation of PHA (Fig. 22.4). The temperature is set in between the 30 °C and 37 °C and pH is maintained according to microbial strains requirements (Kim et al. 2007). The PHA fermentation is employed in two stages: (1) the first stage is the production of high-density microbial culture and (2) the second stage is the enhancement of concentration of PHA, which is usually a nutrient-limited fermentation (Madison and Huisman 1999; Tsuge 2002; Tsuge et al. 2015). The production of PHA in pure culture is suffering from the interference of external nutrients, while in mixed cultures, the production is prompt by the intercellular limitation (Serafim et al. 2008). The production in open mixed culture aids in the cost-friendly system and enhances the efficiency of fermentation (Patnaik 2005; Verlinden et al. 2007; Castilho et al. 2009). When microbial cells are being exposed to the medium with limited nutrient condition for a long period of time results in physiological changes. As the condition goes normal, microbial cells came back to its normal physiological state and PHA formation starts again. This type of fermentation is known as "feast and famine" (Lemos et al. 2008; Daigger and Grady 1982).

Recovery: There are numerous kinds of recovery methods which are used for the synthesis of PHA which are shown in Table 22.6. Among the listed methods, the supercritical fluid (SCF), cell fragility, and use of radiation are the newly introduced methods which are highly yielding than the others. The most common and easy method is biological recovery method. During this process, freeze-dried cells are fed by mealworms and releases fecal pellets which is purified to obtained pure PHA polymers (Surendran et al. 2020).

Table 22.5 List of media used for the production of PHA and its composition

Media name	Composition
Tryptic soya agar (TSA) (40 g/L)	1. Tryptone 17 g 2. Soytone 3 g 3. Dextrose 2.5 g 4. NaCl 5.0 g 5. K_2HPO_4 2.5 g 6. Agar 15 g
Tryptic soya broth (TSB) (30 g/L)	1. Pancreatic digest of casein 17.0 g 2. Papaic digest of soybean meal 3.0 g 3. Sodium chloride 5.0 g 4. Dextrose 2.5 g 5. Dipotassium phosphate 2.5 g
Nutrient agar (NA) (23 g/L)	1. Peptone 0.5 g 2. Sodium chloride 0.5 g 3. Yeast extract 0.2 g 4. Agar 1.5 g
Bifidus selective media agar (BSM) (55.5 g/L)	1. NaCl 3.62 g 2. K_2HPO_4 0.26 g 3. NH_4Cl 0.08 g 4. $NaNO_3$ 0.85 g 5. Na_2SO_4 0.05 g 6. Trace element solution 1.00 mL (a) HCl (25%; 7.7 M) 10.00 mL (b) $FeCl_2.4H_2O$ 1.50 g (c) $MgCl_2.6H_2O$ 3.00 g (d) $CaCl_2.2H_2O$ 0.10 g (e) $ZnCl_2$ 70.00 mg (f) $MnCl_2.4H_2O$ 100.00 mg (g) H_3BO_3 6.00 mg (h) $CoCl_2.6H_2O$ 190.00 mg (i) $CuCl_2.2H_2O$ 2.00 mg (j) $NiCl_2.6H_2O$ 24.00 mg (k) $Na_2MoO_4.2H_2O$ 36.00 mg (l) Distilled water 990.00 mL 7. Vitamin solution 10.00 mL 8. Na-DL-lactate 1.12 g 9. Yeast extract 0.20 g 10. Distilled water 936.50 mL 11. $NaHCO_3$ (8%) 52.50 mL

22.5 Challenges in the Production of Bioplastic Polymers

22.5.1 Cost

The production of bioplastic polymers is highly expensive as compared to conventional plastics. It is a novel technology which is still being in research and development for further understanding (Pathak et al. 2014).

Fig. 22.4 Bioplastic production using PHA

Table 22.6 PHA production recovery methods, its classifications, and materials used for this purpose

Recovery methods	Subdivisions	Materials used	References
Chemical	• Simple digestion • Solvent extraction • Hypochlorite digestion • Enzymatic digestion – Heat treatment – Enzymatic hydrolysis – Surfactant washing • Dispersion process	*Solvents*: • Chloroform • 1,2-Dichloroethane • Methyl ethyl ketone (MEK) • Methyl isobutyl ketone • Ethyl carbonate • 1,2-propylene carbonate • Methanol • Ethanol	Ramsay et al. (1990), Jiang et al. (2006), Fiorese et al. (2009), Lafferty and Heinzle (1978), Tamer et al. (1998), Kunasundari and Sudesh (2011), McCool et al. (1996), Page and Cornish (1993), Hahn et al. (1993), Saharan et al. (2011), Choi and Lee (1997), Holmes and Lim (1990), Berger et al. (1989), Kelly and Muske (2004), Możejko and Ciesielski (2013), Yu and Chen (2006) and Divyashree and Shamala (2010)
Mechanical	• Bead mill • High-pressure homogenization	–	Middelberg (1995), Geciova et al. (2002), Kelly and Muske (2004), Tamer et al. (1998) and Divya et al. (2013)
Biological	• Mealworms • Rats	–	Murugan et al. (2017), Ong et al. (2018a, b) and Kunasundari et al. (2017)
Supercritical fluid	–	Supercritical carbon dioxide	Hejazi et al. (2003), Khosravi-Darani et al. (2003) and Darani and Mozafari (2010)
Cell fragility	–	–	Kunasundari and Sudesh (2011)
Radiations	–	Gamma rays	Bhattacharya (2000)

22.5.2 Threat to the Environment

Since majority of bioplastics are synthesized from hybrid plant biomass like corn, sugarcane, soybean, etc., some of which are toxic for both human and animal use. The production of bioplastics utilized massive amount of woods that results in deforestation and less consumption to humans and animals (Shah et al. 2008).

22.5.3 Misconception

Proper landfilling areas are required for its degradation. It is nonrecyclable and can release greenhouse gases upon disintegration. Some bioplastics need some conditions for its decomposition like for some incineration technique can be employed or others make it with different processes. Bioplastics are dumped at landfill areas which not only take the large area but also proper management for its disposal and decomposition (Mohanty et al. 2002; Mohapatra et al. 2017).

22.6 Applications of Bioplastics

22.6.1 Application in Automobile

(a) Due to the fast-growing population, the high literacy and development rate leads to make the working methods easy and less time consuming. For this purpose, car development become necessary (which comprises of 20% of plastics which are almost 100–150 kg per car weight) (Rusu et al. 2011; Pilla 2011).

(b) The automotive industries need the composite materials which fulfill its properties like ultimate breaking force and elongation, flexural and impact properties, fogging, etc. that are being attained by employing different kinds of bioplastics (Bledzki et al. 2010).

(c) The first car comprises of bioplastics was claimed by Toyota which uses sugarcane biomass-based PET in interior layers in 2015 (Prodanović and Milutinović 2017).

(d) The famous automotive company Ford in Germany uses kenaf reinforced PP composited at the door panel and kenaf fibers in their model “Ford Mondeo” from Bangladesh (Guettler 2009).

(e) Ford Motor Company cars comprise of 75% soybean-based foam in their headset (So 2012).

(f) Daimler AG, a renowned company, uses abaca fiber in the Mercedes exterior components (Bledzki et al. 2007, 2008).

(g) Mitsubishi Company claimed to use the PBS bioplastic on the interior components in February 2006 employing bamboo fibers (Schut 2003).

(h) Volvo Company also employed different soya-based bioplastics along with natural fibers in their seats (Prodanović and Milutinović 2017). The top ten

Table 22.7 Top ten automobile company names, their location, model names, and bioplastic-based components

Company names	Country	Model names	Bioplastic-based components
Toyota Motors	Japan	• Harrier • Celsior • Brevis • Raum	• Floor mats • Door panel • Seat backs • Spare tire cover
Volkswagen Group	Germany	• Passat Variant • Bora • Golf MK4 (A4)	• Seat back • Door panel • Boot liner • Boot lid finish panel
Hyundai Motor Group	South Korea	• FCEV	• Seats fabrics • Starring • Door panel
General Motors	United States	• Chevrolet Trailblazer • Cadillac Deville	• Seat backs • Cargo area floor
Ford Motors	United States	• Focus • Freestar • Mondeo CD 162	• Door panel • B-pillar • Floor trays • Boot liner
Nissan Group	Japan	• EV	• Door parts • Dashboard
Honda Motors	Japan	• Pilot	• Cargo area
Fiat Chrysler Automobiles	Italy/United States	• Marea • Brava • Punto • Alfa Romeo 146, 153	• Door panel
Renault	France	• Twingo • Clio	• Rear parcel shelf
BMW Group	Germany	• 3, 5, 7 series	• Door panels • Seats back • Noise installment • Boot linings • Headliner panels

company names, their models, and their bioplastic components are shown in Table 22.7.

22.6.2 Application in Agro-Textile

(a) Agro-textile includes shade nets, bird nets, insect nets, mulch mats, harvesting nets, rain protection nets, fishing nets, etc. (Neveu and Gregoire 1991).
(b) Shade nets are the agro-textile products derived from the threads of polyethylene or polypropylene (Cărpuş et al. 2018).

(c) Insect nets are the nets derived from polyethylene woven monofilament mashes which are used to trap insect and are also known as anti-insect nets (Mandle 2019).

(d) Bird nets are the nets made up of polypropylene or high-density polyethylene (HDPE) and employed to minimize fruit loss caused by birds and are also known as anti-bird nets (Restrepo-Osorio et al. 2019).

(e) Windshield nets are the nets employed to avoid crop losses from strong wind or windstorm and are made of bioplastics (Jiang et al. 2020).

(f) Mulch mats are made from biodegradable mulch like jute and are employed to suppress weed growth (Ghosh et al. 2014).

(g) Harvesting nets are the bio-based nets made by using warp knitting technique and employed to avoid fruits falling from trees (Gangopadhyay and Hira 2010).

(h) Fishing nets are also one of the most common bio-based products employed to catch fishes from seas, rivers, oceans, etc. (Scarlat et al. 2017).

(i) Rain protection nets are the bio-based nets employed to avoid severe rain losses or to protect high-water-sensitive crops or plants (Restrepo-Osorio et al. 2019).

22.6.3 Application in Mulching Films

(a) Usage of mulching in agricultural activities helps in soil moisture protection and crop protection and enhances crop yield (Kasirajan and Ngouajio 2012; Rydz et al. 2015).

(b) Plastic mulch is composed of primarily low-density polyethylene (LDPE), high-density polyethylene (HDPE), and linear LDPE (Kasirajan and Ngouajio 2012; Sintim and Flury 2017).

(c) Danimer Scientific accommodates a patent named Nodax™ which is a biodegradable copolymer of PHA, a commercially available agricultural mulch (Hassan et al. 2006; Bond and Noda 2006).

(d) Metabolix Inc. produces Mirel™ (PHA-based polymer) resin which is also an agricultural mulch (Andrews 2014; Castilla-Archilla et al. 2019).

(e) Like the above patent agro-based mulches, there is other patents bio-based material that can be employed as mulching materials such as US20130029124A1, WO2001093678A2, KR20040071992A, and CN102140185A produced by BASF SE, The Procter & Gamble Company, SK Chemicals Co. Ltd., and Ruide Yu, respectively (Loos et al. 2015; Noda and Satkowski 2005; Amelia et al. 2019).

22.6.4 Application in Food Packaging

(a) Bioplastic polymers are employed as a packaging material in different industries for increasing the shelf life of the goods (Rahman et al. 2019).

(b) These polymers are biodegradable and used to maintain the quality of the product (Bilo et al. 2018).

(c) It aids in conserving the nutritional content of the products.
(d) It also can be used in the making of agricultural tools like sprayers, cans for watering in the garden, etc. (Malinconico 2017).
(e) It reduces the problem of disposal of the wastes (Calabrò and Grosso 2018).
(f) It sorts out the municipal solid waste management problems (Emadian et al. 2017).
(g) It can be employed for long time storage of the product (Thakur et al. 2020).
(h) It can minimize the danger of microbial attack (Yamane 1992).
(i) It has high thermal degradation properties to avoid damage to the product from high temperature (Sudesh and Iwata 2008).
(j) It is basically hydrophobic so therefore reduces the moisture barrier property (Avella et al. 2005).
(k) Additives used in the production of bioplastics like plasticizers, UV stabilizers, natural pigments, etc. aid in maintaining its quality (Weber 2000).
(l) Development of bioplastics sorts out the menace of disposal of plastics which aids in the bio-assimilation of plastics (Ghalem and Mohamed 2008; Chatham 1996; Briassoulis 2004).
(m) It can be used in medical field as drug nano-carriers, nano-medicines, device for tissue engineering and implant in vitro, etc. (Lyu et al. 2016; Maya-Vetencourt et al. 2017; Sung et al. 2019; Lee et al. 2020; Greenwood et al. 2018; Cacciotti et al. 2018; Lei et al. 2017; Rand et al. 2018).
(n) Reduction of greenhouse gases (GHGs) in the production of plastics can be attained by employing bio-based materials (Jariyasakoolroj et al. 2018).
(o) PHB acts as a nucleating agent, which enhances the mechanical resistance and barrier properties (Arrieta et al. 2017).

22.7 Bioremediation

Bioremediation is the problem of employing microorganisms to clean up the environment and aids in the removal of hazardous contaminants from the environment (Strong and Burgess 2008; Zeyaullah et al. 2009). This process relies on promoting microbes growth on the contaminant sites that can be done by several ways like introduction of indigenous microbes or maintenance of environmental conditions for microbial consortia growth (Hess et al. 1997; Smith et al. 1998; Agarwal 1998). In the bioremediation process, microbes employed contaminants as their energy source (Tang et al. 2007). The general environmental condition needed for bioremediation as given by Vidali (2001) is shown in Table 22.8. Microbial activity is highly dependent on pH, temperature, and moisture contents. Microbial activity is also depending upon oxygen requirements, whether it is aerobic or anaerobic because anaerobic microbes best work at anaerobic and aerobic microbes at aerobic condition (Allard and Neilson 1997). Most bioremediation process occurs at aerobic condition, but can also be done at anaerobic condition by permitting degradation of recalcitrant molecules (Colberg and Young 1995). There are different types of bioremediation known, but the main types are in situ and ex situ bioremediation.

Table 22.8 Environmental and nutritional conditions needed for bioremediation

Parameters	Bioremediation requirements	Parameters	Bioremediation requirements (%)
Soil pH	5.5–8.5	Iron	0.2
Soil moisture	20–28%	Potassium	1
Temperature	15–45 °C	Magnesium	0.5
C:N ratio	10:1	Calcium	0.5
Oxygen contents	Aerobic: Minimum 10%	Sodium	1
Heavy metals	200 ppm	Hydrogen	8
Soil type	Low clay or silty	Sulfur	1
Nutrients	Depending on microbe requirements (but generally listed below)	Chloride	0.5
Carbon	50%	Others	0.3
Nitrogen	14%	Contaminants	Not too toxic
Phosphorus	3%	–	–

22.7.1 In Situ Bioremediation

In situ bioremediation is a biological process where microbes are employed to metabolize organic contaminants into inorganic products under natural or artificial conditions (United States Department of D 1997; Potter 1996; Schinner and Margesin 2005). It is also known as natural attenuation or intrinsic bioremediation because of its natural occurring without any special process (Kao et al. 2006; Kao and Prosser 2001). This process depends on the pollutant sources, concentration, chemistry, toxicity, transport, solubility, volatility, dispersion, adsorption, detection, determination, monitoring, physic-chemistry and biology of groundwater, environmental conditions, nutrient sources, presence of electron acceptor, environmental standards' limitation for water and soil, hydrology and hydrogeology of contaminant sites, biodegradability of contaminants, microbiological conditions, and chemistry and mechanics of soil at contaminant sites (Andreoni and Gianfreda 2007; Boopathy 2000; Farhadian et al. 2006; Kampbell et al. 1996; MacDonald et al. 1999; McGuire et al. 2005; Schreiber and Bahr 2002). It is further classified into three kinds on the basis of land treatments.

22.7.1.1 Bio-Venting

Soil venting is defined as the extraction of soil vapors at particular sites to remediate soil from lower relative molecular weight contaminants (Hinchee et al. 1987; Ardito and Billings 1990). Bio-venting is the combination of soil venting and bioremediation which employs oxygen for the biodegradation of contaminants in the presence of indigenous microbes (Dupont et al. 1991; Lee and Swindoll 1993; Michael and Swindoll 1995). It aids in the degradation of simple hydrocarbons and low molecular weight contaminants which are deep under the soil (Hinchee et al. 1991).

22.7.1.2 Bio-Augmentation

Bio-augmentation is the remediation technique during which indigenous microbes are employed at the contaminant sites (Vogel 1996). The growth of these exogenous microbes can be limited by the competition with the nonindigenous species and the presence of other indigenous microbes due to long time period degradation at that site (Vidali 2001). There are new approaches introduced in this field which are (1) bio-augmentation with carrier encapsulated cells (Gentry et al. 2004); (2) gene bio-augmentation where genetic engineering employs to transfer particular remediation gene into microbes (Pepper et al. 2002); (3) rhizosphere bio-augmentation which is the inoculation of microbes in rhizosphere near the plants (Andreazza et al. 2010); and (4) phyto-augmentation which is the transfer of gene into the plant without employing any kind of microbial inocula (Redfern and Gunsch 2016; Kumar and Saxena 2020).

22.7.1.3 Bio-Sparing

Bio-sparing is the process of injecting oxygen (air) into the groundwater for the biological disintegration of contaminants by the microbes (Chibuike and Obiora 2013, 2014; Bennett 1999). There are two factors which primarily affect the bio-sparing process: the pollutants' biodegradability and permeability of polluted soils (Waudby and Nelson 2004; United States Environmental Protection Agency. Office of Solid W, Emergency R, Environmental Management S 1997).

22.7.2 Ex Situ Bioremediation

Ex situ bioremediation is the technique of remediation of soil and groundwater via removal by excavation (soil) or pumping (water) (Tomei and Daugulis 2013; Brar et al. 2006). The contaminants are being excavated from the soil to degrade aerobically in the existence of microbes (Jovančićević et al. 2008). There are two main categories of ex situ bioremediation.

22.7.2.1 Slurry Phase Bioremediation

It is basically a process of mixing three phases that is liquid, solid, and gas to degrade water-soluble pollutants aerobically. It is also known as bioreactor or slurry bioreactor. In this method, engineering containment system employed for processing polluted solid and liquid wastes (Reinhart and Townsend 1997; Reinhart et al. 2002).

22.7.2.2 Solid Phase Bioremediation

Solid phase bioremediation is the technique in which the polluted solid wastes are placed into piles that includes organic wastes. These wates are biodegraded by microbes and proper ventilating it by turning the piles (Mueller et al. 1991; Nano et al. 2003). This technique includes the composting process, biochar, land farming, and soil biopiles.

22.7.2.3 Composting

Composting is the biodegradation of organic wastes either aerobically or anaerobically (Smith et al. 2001). It is the method alternative to commercial solid waste management (Tweib et al. 2011). It has four stages: mesophilic, thermophilic, cooling, and curing phase (Hoitink and Boehm 1999).

22.7.2.4 Land Farming

It is an ex situ technique, in which the excavated soils are spread on the prepared beds to degrade contaminants aerobically. In this method, nutrients and minerals are added which help in enhancing microbial growth (Poland et al. 2008; Atlas and Philp 2005; Paudyn et al. 2008).

22.7.2.5 Soil Biopiles

Soil biopiles are the remediation technique employed to remediate soils with petroleum contents. In this method, the excavated soils are placed in the pile which is degrading either aerobically or by application of nutrients and minerals for the microbes. It is also known as biocells (Płaza et al. 2005; Iturbe et al. 2004).

22.7.3 Enzymes Used in Bioremediation

Bioremediation is the microbes mediated technique which converts hazardous wastes into nonhazardous materials (Vidali 2001; Leung 2004). Enzymes are the biological catalysts which are employed in the conversion of substrate into product (Lehninger et al. 2004). Oxidoreductases and hydrolases class of enzymes are employed for bioremediation (Karigar and Rao 2011). Among oxidoreductases subdivisions, oxygenase (oxidation of reduced substrate by supplying oxygen from molecular oxygen) (Fetzner and Lingens 1994; Fetzner 2002; Arora et al. 2009), peroxidase (oxidation of lipids and phenolic compounds by utilizing hydrogen peroxide) (Hiner et al. 2002; Koua et al. 2009), and laccase (oxidation of phenolic compounds and aromatic compounds into water) (Bollag et al. 1998; Giardina et al. 1995; Rezende et al. 2005) are only employed for bioremediation. Among hydrolases enzymes, lipase (Riffaldi et al. 2006; Margesin et al. 1999), cellulase (Bennett 1999; Adriano-Anaya et al. 2005; Rixon et al. 1992), and protease (Singh and Devi 2019; Beena and Geevarghese 2010) are used.

22.7.4 Role of Bioplastics in Bioremediation

(a) Release of low greenhouse gases upon degradation (Benn and Zitomer 2018).
(b) An environmentally friendly system (Piemonte 2011).
(c) Helps in low carbon emission (Maheshwari et al. 2013).
(d) Aids in waste management by using numerous techniques like composting, biochar, etc.

(e) It is biodegradable and so easily decomposed and also used as landfill (Kalia et al. 2000b).
(f) Most of the renewable sources are employed which are nonhazardous (Calabrò and Grosso 2018).
(g) Reduced environmental pollution (Sabbah and Porta 2017).
(h) Its production leads to less use of fossil fuel or petroleum materials (Gill 2014).
(i) Upon biological decomposition, some other contaminants like persistent organic pollutants (POPs) exits in soils or water are biodegraded together by microbes (Marjadi et al. 2010).
(j) Less intrusion of microbes with food products (Jabeen et al. 2015).
(k) Enhances food sustainability (Pathak et al. 2014).

22.8 Conclusion and Future Prospects

With the rapid awareness of employing hazardous nonrenewable materials, there is a demand of alternate that originates from renewable resources and are biodegradable. Bioplastic is one of the most interesting materials that fulfills this condition but with some limitation that can be edited by using plasticizers or additives. Its production is cost-effective at first stage; that is why it is not easily adopted by underdeveloped countries like Pakistan. Nowadays, microbiology and genetic engineering is used in this field to promote this technology. New approaches should be developed which will overcome its disadvantages. Further, research and development in this field is the need of human for the benefit of the environment. Industrial development and proper strategies play a crucial role in environmental sustainability.

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Challenges of Bioplastics as Bioinks for 3D and 4D Bioprinting of Human Tissue-Engineered Structures

23

Sherif S. Z. Hindi, Uthman M. Dawoud, and Mona O. Albureikan

Abstract

Tissue engineering (TE) is an important field of regenerative medicine for repairing deteriorated human tissues. Its ordinary techniques have encountered some limitations such as manual intervention and they are inconsistent and inflexible throughout the varies medical cases as a technique as well as the quality of the final scaffold. The 3D- and 4D- bioprinting technologies (BTs) constitute an advanced branch of the TE used to fabricate prosthetic tissue scaffolds imitating the parent tissue properties. The BT depends essentially on so-called stem cells incorporated in bioplastic polymers termed as bioink and growth factors. The stem cells are essential for the TE due to their ability to differentiate into several types of functionally different cells according to the stimuli affecting them. Generally, the BT technique performs the layer-by-layer manner for depositing bioink to create tissue-like structures for the TE applications. This is done using certain devices called bioprinters that can be classified into three principal types, namely inkjet-, extrusion- and laser-assisted bioprinters. Bioink is the cornerstone of the BT and can be classified into four types, namely structural, sacrificial, functional and supportive. Many non-biodegradable materials such as ceramics and metals have been utilized for the TE applications to enhance tissue adhesion,

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especially load-bearing forces. On the other hand, although natural bioplastics are generally biocompatible, biodegradable and bioactive, they did not possess adequate mechanical characteristics essential for high-load-bearing applications. Some lights were shed on mimicking specific human tissues, including, but not limited to, myocardial treatments, auricular cartilage for knee joint, liver-mimetic honeycomb, bone health and regeneration, osteochondral tissue, human neuroblastoma, cancer tumour and fat grafting.

Keywords

Tissue engineering · Bioplastic · Hydrogel · Cellulose nanofibrils · Bioink · Stem cells · 3D bioprinting · Bioprinters

23.1 Introduction

The global demand for tissue regeneration as well as organ replacement is increasing progressively. The great gain in this medical field was achieved by using 3D bioprinting. The crucial component of this process is the bioink with certain properties. Applying either single or hybrid techniques is required to develop more suitable bioink for the 3D printing of a bio-tissue (Gopinathan and Noh 2018; Gungor-Ozkerim et al. 2018). During bioprinting, a bioink solution containing desired living cells is allowed to be cross-linked and cured immediately to regenerate the desired tissue model. Bioinks may be prepared from natural or synthetic single biomaterial or a homogenized hybrid material. An ideal bioink must have suitable mechanical, rheological and biological characteristics that are essential to adapt the parent functionality of the target tissues (Gungor-Ozkerim et al. 2018). The goal of this chapter is to feature recent advances in the tissue engineering field performed using 3D and 4D bioprinting, focusing on developing bioink and bioprinting methods and their applications, as well as the use of natural and synthetic additives which provide unique, novel and critical functionalities of the mimicked tissues.

23.2 Human Cells

There are several types of cells in the human body (Fig. 23.1). They can be divided into three categories according to their origin belonging to endoderm, ectoderm and mesoderm (Wainwright 2010). In each germ layer, cells are differentiated into tissues as well as embryonic organs. The native tissues have complex structures composed of different cell types and extracellular matrices of materials and biomolecules. Neither strategies nor protocols of ordinary tissue engineering possess the ability for regeneration of biomimetic and heterogeneous tissue constructs due to the lack of suitable biomaterials and technologies (Ashammakhi et al. 2019).

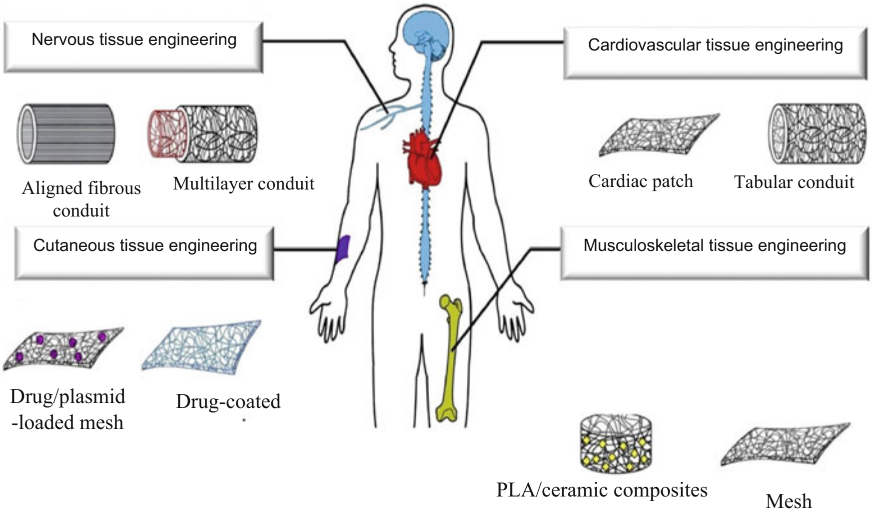

Fig. 23.1 Isolated cells, tissue-inducing substances, scaffolds

23.2.1 Endoderm

It is the innermost layer of the triple layer embryonic germ. In zebrafish, it results in the lining of the gut, gills, liver, pancreas and gallbladder as well as the pharyngeal pouches. The cell types in this layer are exocrine secretory epithelial cells, barrier cells and hormone-secreting cells (Goessling and Stainier 2016).

23.2.2 Ectoderm

The ectoderm is the most exterior of the three primary germ layers formed in the very early embryo (Wainwright 2010). There are several types of its cells, namely exocrine secretory epithelial cells, hormone-secreting cells, epithelial cells, oral cells and nervous system (sensory transducer cells, autonomic neuron cells, sense organ and peripheral neuron-supporting cells, central nervous system neurons and glial cells and lens cells).

23.2.3 Mesoderm

In all bilaterian animals, the mesoderm is one of the three primary germ layers arisen in the third week of embryonic development for all bilaterian animals. It is formed through a process called gastrulation. There are three important components: the paraxial mesoderm, the intermediate mesoderm and the lateral plate mesoderm. Different cells are differentiated from this layer into metabolism and storage cells, secretory cells, barrier cells (urinary system, reproductive system and circulatory system), extracellular matrix cells, contractile cells, blood and immune system cells, germ cells, nurse cells and interstitial cells (Ruppert et al. 2004; Wainwright 2010).

23.3 Tissue Engineering

Several synthesis techniques concerning to ordinary chemical engineering techniques and additive manufacturing (AM) are utilized to fabricate tissue engineering (TE) scaffolds (Fig. 23.1). These ordinary techniques are encountering some limitations such as manual intervention, inconsistent and inflexible processing techniques as well as the quality of the final scaffold such as inconsistent permeable structure (size, geometry and distribution) of pores (Vaezi et al. 2018). The impacts of the TE strategies and tools on clinical practice to prevent viral outbreak are illustrated in Fig. 23.2. The top-down route has been performed for assembling scaffolds at micrometric scale (μm), while the bottom-up route has also been applied for assembling small groups of atoms or molecules at the scale of nanometres (nm) to reach micrometric scale constructs. Any of them can be applied to fabricate TE scaffolds as shown in Fig. 23.3.

23.4 The Bioprinting

The 3D- and 4D-bioprinting technology involves the use of raw materials that contain living cells to mimic deteriorated tissues or organs. The classification of bioprinting techniques used for tissue engineering applications is presented in Fig. 23.4. New biofabrication technologies have developed and applied including lithography-based techniques such as stereolithography, digital light processing,

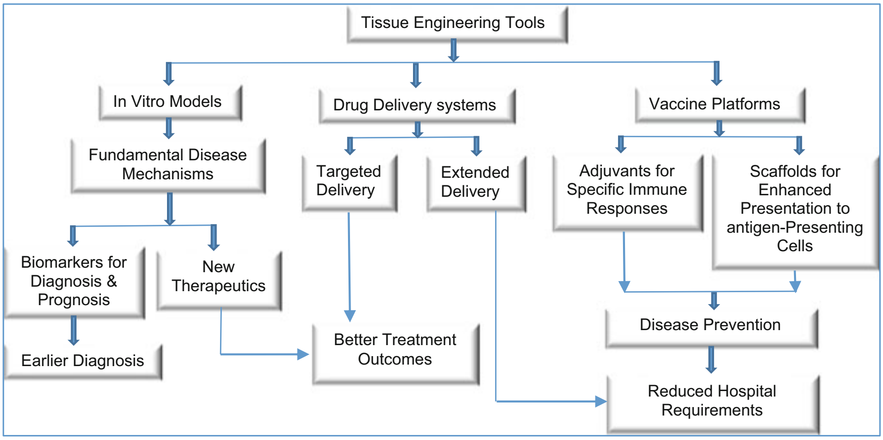

Fig. 23.2 Impacts of tissue engineering tools on clinical practice to prevent viral outbreak

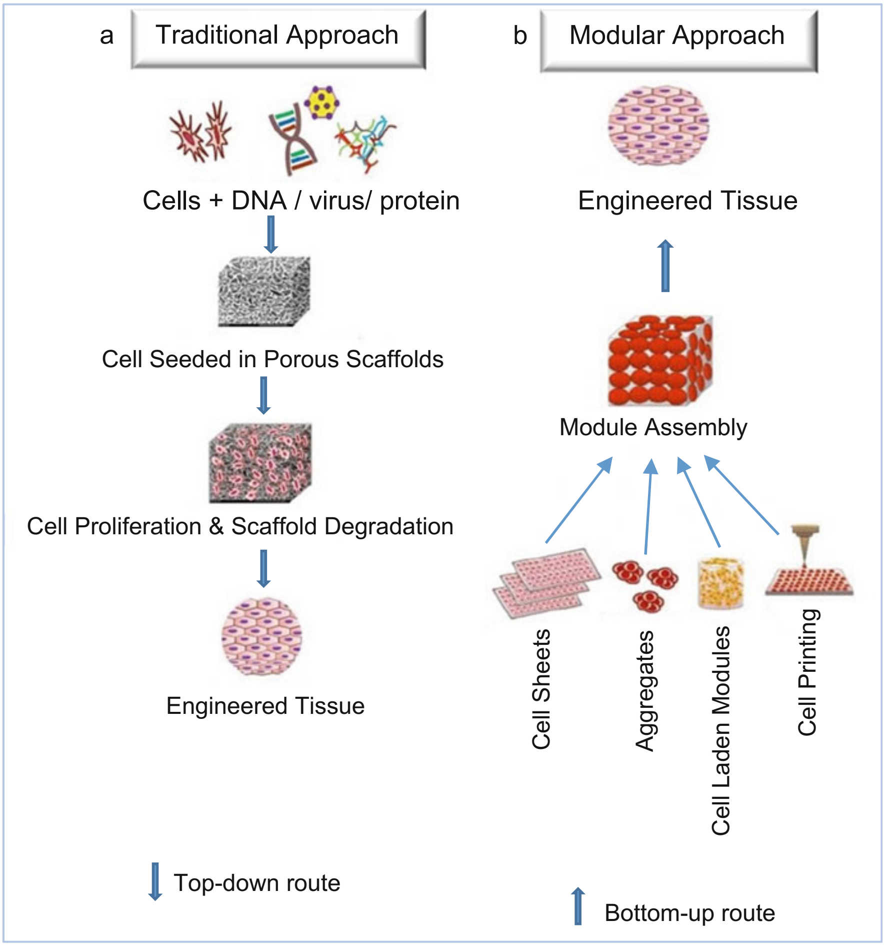

Fig. 23.3 The routes used in tissue engineering: (**a**) top-down and (**b**) bottom-up

continuous liquid interface and two-photon polymerization, which allow for the fabrication of 3D structures with high spatial resolution (Lin et al. 2013; Qin et al. 2014; Ovsianikov et al. 2014; Van Hoorick et al. 2017; Tromayer et al. 2018; Lim et al. 2018). These approaches involve spatial patterning of light to photo-cross-link-specific regions of a bioink (acting as a resin), usually composed of a low-viscosity photo-cross-linkable hydrogel precursor (Lim et al. 2018; Miri et al. 2018).

23.4.1 The 3D Bioprinting

It is the utilization of 3D-printing technique incorporating cells, growth factors and biomaterials (bioinks) to fabricate prosthetic tissue scaffolds that maximally imitate

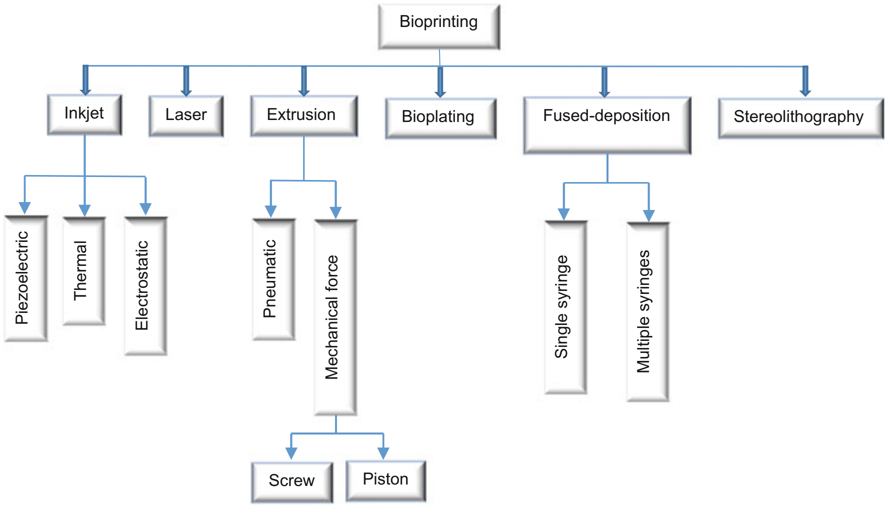

Fig. 23.4 Classification of bioprinting methods used for biomedical applications

natural tissue characteristics (Singh and Thomas 2018; Wu et al. 2018; Mobaraki et al. 2020; Saygili et al. 2020). Generally, this technique performs the layer-by-layer manner for depositing bioinks to create tissue-like structures that finally are helpful in medical as well as TE fields. Significant progress in 3D-bioprinting technology has remarkedly improved either tools or schemes of the manufacturing process. While scaffolds have ordinarily been utilized, 3D bioprinters are increasingly being employed although they are scaffold independent (Ong et al. 2018). The 3D bioprinting has revolutionized the medical industry helping overcome the problems associated to the current TE methods, such as longer treatment times (especially with organ transplants) as well as defencing infectious diseases effectively (Vijayavenkataraman et al. 2016). Among different techniques to produce scaffolds, 3D printing has gained great attention due to its ability to offer good cellular adhesion, proliferation and accelerating regenerations (Sahranavard et al. 2020). Bioprinting will help for either ageing population or paediatric field in which the bioprinted tissue or organ should be able to grow with the patient (Vijayavenkataraman et al. 2016). The essential target of bioprinting is the creation of functional 3D-biomimetic composites for various application areas. Successful fabrication of model tissues is dependent on pivotal parameters including microenvironment conditions, proper scaffold design and cell source precursor. As the cell culture-based assays have vital roles in the biomedical field, bioprinted tissue analogy would provide great chances to investigate, screen and treat diseases. Recent 3D-bioprinting technology helps in print cells and scaffolds simultaneously that offer good chance for investigating several medical cases such as microbial infections, interactions between host and pathogen, niches for microbiota, biofilm synthesis and assessments of microbial resistivity to antibiotics (Saygili et al. 2020).

3D-printing technologies have introduced many advances to the biomedical field such as rapid fabrication of complicated constructs although some limitations including biomaterials diversity and printing methods versatility did not solved completely (Whyte et al. 2019).

Powder inkjet printing is a 3D-printing technology that permits solidifying the powdered material by chemical bonding other than photopolymerization, melting or sintering action. Unfortunately, the liquids associated with organic powders are highly acidic which can affect the printer components limiting this printing technique (Whyte et al. 2019). To enhance the mechanical properties of cellularized scaffolds, Kolan et al. (2020) mixed human adipose-derived mesenchymal stem cells (ASCs) in an alginate-gelatin (1:1) hydrogel mixer which is deposited within a special composite structure. The resultant 3D-bioprinted scaffolds were found to be nonhomogeneous concerning to the cell viability along their thickness. Accordingly, lower viability were noticed at the bottom regimes, while higher viability featured the top layers of the scaffold. Producing high-resolution products is essential for the fabrication of functional organs for transplanting. Increasing the resolution of a 3D-bioprinted scaffold was achieved by decreasing the printing nozzle diameter. However, this may increase the forces which cause cell damage during the printing process (Cahall et al. 2020). They presented a novel cell encapsulation technique that protects living cells from distortion during the extrusion process. The cell encapsulation technique was performed by coating the living cells in proper polymers to maintain their size, shape and mechanical properties after extrusion. However, the shear forces affecting the cells upon extrusion may cause noticed damage that are harmful for the cell membrane integrity and impact the parent cellular functionality (Cahall et al. 2020).

Webb and Doyle (2017) have developed a simple assessment method, namely the parameter optimization index (POI) for the bioprint product-based extrusion method. The aim of this method is to minimize the shear forces affecting the bioink as well as encapsulated living cells. They used a mixture of alginate and gelatin (7% and 8%, respectively), and they extruded the blend through 25-, 27- and 30-gauge print nozzles at 1−6 mm/s print speeds and at 100–250 kPa print pressures. They showed that the optimum print is achieved with a 30-gauge nozzle, 100 kPa print pressure and 4 mm/s print speed. The POI is intuitive and easy to assess and could be a helpful method through a wide spectrum of 3D-bioprinting applications (Webb and Doyle 2017). Recent achievements in the TE field have focused on developing technologies able to provide personalized scaffolds correcting the shape defects that may result in a 3D bio-scaffold. This target can be achieved by combining a suitable bioink for creating a physiological milieu for cell growth (Tonda-Turo et al. 2020). Diaz-Gomez et al. (2020) provided a novel technique to fabricate a 3D-printed multi-material scaffolds by using a robust thermoplastic structure mixed with low-viscosity bioinks. A fibre engraving method was performed to make grooves on the thermoplastic-printed fibre surfaces using a commercial 3D printer, and, subsequently, a low-viscosity bioink was deposited into this groove. They fabricated and characterized a scaffold containing poly(ε-caprolactone) as the thermoplastic polymer and a gelatin-based bioink. The gelatin methacryloyl

(7.5 w/v%) bioink was loaded with either 5 w/v% of poly(lactic-co-glycolic acid) microparticles containing fluorescent albumin or mouse fibroblasts (1 × 10^6 cell/mL). In vitro fabrication of vascular networks is one of the most complex challenges currently faced in the TE field. Heidari and Taylor (2020) described a method to fabricate multi-layers of cell-laden microstructured hydrogel featured with coaxial geometries and heterogeneous elastic moduli. The technique can be used for fabricating in vitro vascular networks incorporated in physiologically realistic hydrogels. Their technique helps to eliminate rigid polymeric surfaces from the vicinity of the cells. This can solve an important problem facing many microfluidic models and allows layers of multiple cell types to be integrated (Heidari and Taylor 2020).

The invention of 3D bioprinting will make a revolution concerning the field of TE and regenerative medicine. Markstedt et al. (2015) made a wonderful bioink from cellulose nanofibrils (CNF) and alginate for the 3D bioprinting of living soft cellular tissue. The novel bioink possesses the superior shear-thinning characteristics of CNF with the fast cross-linking curing behaviour of alginate. In addition, evaluating printability concerning printer parameters and shape fidelity revealed that the bioink enabled bioprinting of both 2D-gridlike structures and 3D constructs. It was indicated by Müller et al. (2017) that finding effective bioinks is an important challenge of bioprinting in order to enhance cell growth, tissue maturation and formation of functional grafts. They applied bioink composed from alginate sulphate and nanocellulose which showed good printability when using small-diameter nozzles and valves.

23.4.2 The 4D Bioprinting

The 4D bioprinting is a new technology having a potential to enhance the 3D-bioprinting field by confounding an additional dimension of time (Ong et al. 2018). Several bioinks were found to be sensitive to external stimuli, whereby their shape, structure, function and other behaviours could be changed when they received a stimuli. It can be used in many TE applications such as muscle tissue by applying a repeatedly or pulse electric stimulation to the bio-construct that can affect the bioink. However, this stimulus can be applied by a bioprinted nerve. In addition, the 4D bioinks must be self-assembled into the target bio-construct and when it is supplied with nutrients during culturing for a proper duration, it produces a 3D-construct suitable for implantation. Activity of the living stem cells incorporated in the bioink may be influenced by the concentration of nutrients added as well as those molecules formed due to metabolic activities of these cells. For instances, these materials can be released sensitively to the specific cell phenotypes or when a phenotype-specific protein reaches a certain concentration level. Cellularized scaffolds fabricated using hydrogels do not have adequate strength to act as stand-alone implant devices for repairing and regeneration of hard tissues. Auditioning thermoplastic polymer to bioink recipe can support the resultant scaffold structure by supporting its integrity to scaffolds, while cells and growth factors in the hydrogel formula offer biological stimulation for the tissue fabrication (Kolan et al. 2020). Tonda-Turo et al. (2020) have introduced a novel chitosan-based hydrogel formula by confounding photo-cross-linking and thermo-sensitive properties together. Commercial chitosan was primarily methacrylated and then mixed with β-glycerol phosphate (β-GP) to introduce the thermally induced phase transition. It was indicated by Ji et al. (2020) that norbornene-modified cellulose-based bioink has a significant potential to fabricate 3D-bioprinted constructs considering the increased complexity of the product. In addition, for the bioink precursors, the high abundance, low cost and ability to selectively tether molecules or control cross-linking properties also increase the importance of this bioink formulation. Moses et al. (2020) have explored a multi-functional silk-based cartilage and bone bioinks for fabricating complicated TE constructs including osteochondral interface. The 3D-bioprinted osteochondral interface made from hydrogel appeared good permeability. It was demonstrated that strontium-based nanocomposite bioinks designed for extrusion-based 3D bioprinting of osteoconductive scaffolds have retained their shapes at long run with promising applications for bone tissue regeneration (Alcala-Orozco et al. 2020). Furthermore, Triyono et al. (2020) studied the mechanical properties of bone graft biocomposites of bovine hydroxyapatite (BHA)-glycerin by deposition using 3D printer. Isosorbide-derived polyesters containing different amounts of double bonds were synthesized by Lammel-Lindemann et al. (2020) for better cross-linking and/or functionalizing the bioink precursor. After thermal initiation and photo-cross-linking, the bioink was mixed with a photoinitiator, co-cross-linker and solvent to fabricate the 3D-bioprinted scaffold.

23.5 The Bioprinters

The 3D bioprinter is a device that mimics complex structures from the bottom to up routes in the three cardinal directions (Markstedt et al. 2015). There are three principal types of printers commercially available for the 3D bioprinting, namely inkjet-, extrusion- and laser-assisted bioprinters. Inkjet printers are essentially used in bioprinting for fast and large-scale production (Fig. 23.5). One type of inkjet printer, termed as drop-on-demand inkjet printer, has the ability to print bioblends in exact quantities, reducing cost and waste. Printers that utilize lasers are able to produce high-resolution printing qualities. A standard trademark of bioprinter known as BIO X has been trusting by revolutionizing fields such as TE, drug discovery and toxicity research and enabling scientists to perform more in 3D-cell culturing faster. The bioprinter printhead units (BPU) as shown in Fig. 23.5 are varied widely from standard pneumatic electromagnetic BPU, droplet BPU, temperature-controlled pneumatic BPU (4 °C to 60 °C), syringe pump BPU, thermoplastic BPU, photocuring BPU to HD camera tool head. In addition, they are compatible with standard petri plates, multi-well plates as well as custom inserts for a wide spectrum of bioinks. It has also a modern technology for clean chamber with UV-germicidal lamps, dual-filter system and exchangeable photocuring modules (365 nm and 405 nm) as presented in Fig. 23.5.

Fig. 23.5 Different models of the bioprinters used for the 3D bioprinting of the human tissue engineering: (**a**) extrusion 3D bioprinters and (**b**) light-based 3D bioprinters (extracted from www.cellink.com)

23.5.1 Extrusion Bioprinters

Extrusion printers can mimic living cell matrices layer-by-layer to fabricate 3D constructs and may use hydrogels infused with cells. There is a recent trend for the fabrication of TE scaffolds either directly by using 3D printing or indirectly by making negative scaffold to be used as a mould using the extrusion bioprinters. The reasons of the widespread use of this technique are due to their ability to process variant biomaterials, their possibility to fabricate scaffolds in a cell-friendly environment, their high reproducibility and flexibility and their simple process control. In addition, there is a new solvent-based extrusion free forming method for printing bioceramic scaffolds which is useful to make polyether-ether-ketone-based composites (Vaezi et al. 2018).

23.5.2 Light-Based Bioprinters

Light-based 3D-printing methods offer exquisite control over material structure. However, the use of these methods for structuring hydrogels is underdeveloped. In particular, the ability to print hydrogel objects containing internal voids and channels is limited by the lack of well-characterized formulations that strongly attenuate light and the lack of a theoretical framework for predicting and mitigating channel occlusion (Benjamin et al. 2019). Light-based 3D-printing methods can be used to form objects much faster and with finer spatial resolution than FDM printers (Melchels et al. 2010; Tumbleston et al. 2015). These printers rely on photopolymerization to initiate the polymerization of a liquid into a solid. This can be achieved by projecting a 2D digital light processing (Zhang et al. 2012; Gauvin et al. 2012) or by raster-scanning a beam of laser light stereolithography (Melchels et al. 2010; Jacobs 1992) into a photo-cross-linkable liquid.

23.6 Bioplastics

Many non-biodegradable materials such as ceramics and metals have been utilized for the TE applications to enhance tissue adhesion, especially load-bearing forces. On the other hand, although natural polymers are generally biocompatible, biodegradable and bioactive, they did not possess adequate mechanical characteristics essential for high-load-bearing applications (Whyte et al. 2019). Chitosan, cellulose, Pluronic P-85, hyaluronic acids, agar-agar, alginate, gelatin and collagen are types of natural polymers that can provide denser and higher-quality constructs than those made by ordinary bioink counterparts. However, some limitations are accompanied to many biopolymers like nonresponsive to light or thermal degradation at relatively lower temperatures, thereby reducing their performance in many 3D-printing applications (Whyte et al. 2019). Figure 23.6 contains the chemical structures of the most famous polymers used in the TE field. Chitosan is one of the best biopolymers for the TE and regenerative medicine because of its acceptable characteristics such as hydrophilicity, biocompatibility, easy biodegradability and active antimicrobial activities (Sahranavard et al. 2020). Microcrystalline cellulose (MCC) and nanocellulose including nanocrystalline cellulose (NCC) and cellulose nanofibrils (CNF) have many advantages to be used in biomedical applications including reinforcing transparent wound dressings. The material is strong, allows for the production of transparent films and can form elastic gels having bio-responsive properties (Rees et al. 2015).

The small nanometric dimensions of the CNF (20–200 nm) produce lower viscosity of the TE composite, thus yielding a bioink with good rheological

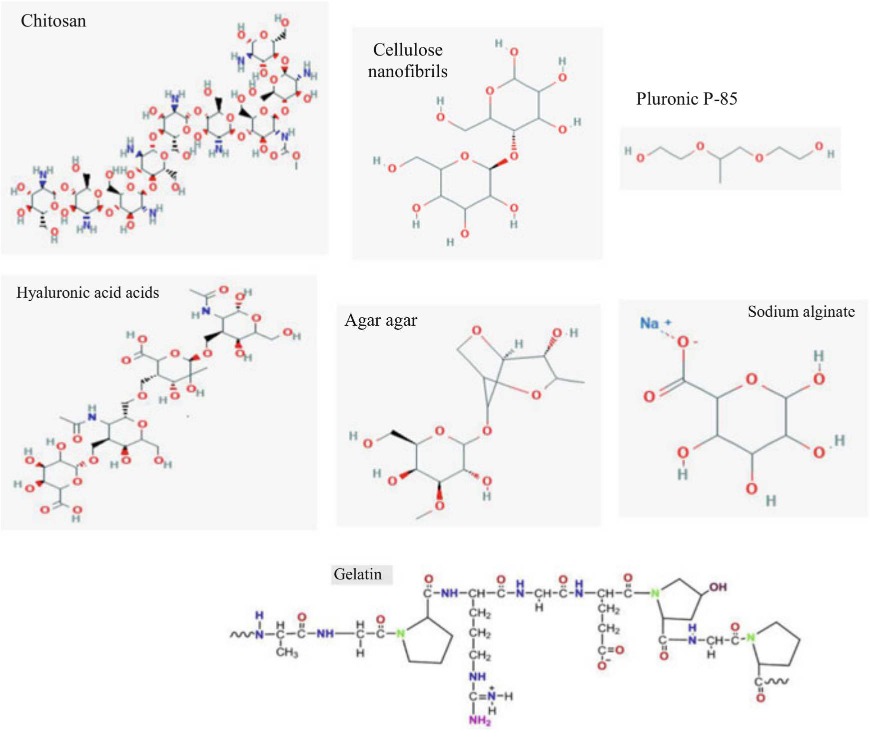

Fig. 23.6 Chemical structure of important bioplastics (extracted from https://pubchem.ncbi.nlm.nih.gov/)

properties for 3D printing porous structures having antibacterial activities (Rees et al. 2015). In addition, the nanocellulose-based bioink was found to be a suitable hydrogel for 3D bioprinting of living tissues and organs (Markstedt et al. 2015). It was indicated by Bedell et al. (2020) that the collagen- and gelatin-based bioinks give TE composites having the highest viability, metabolic activity and prominent cell spreading within the bioink droplet culture when compared to the other bioinks.

23.7 Hydrogel

Hydrogels are known to be water-based gels or hydrophilic substances constituted of polymer dissolvable in water and can be cross-linked, consolidated and, finally, solidified (Peppas et al. 2000; Drury and Mooney 2003; Qiu and Park 2001). Furthermore, since they contain a lower mass content, they are softer and can be easily swelled and deformed compared to the other polymers. In addition, hydrogels vary widely in their compatibility with biological systems (Zhu 2010) and, subsequently, in their medical applications (Bowman and Kloxin 2008), medicine (Zhu et al. 2015; Slaughter et al. 2009; Bae et al. 2010; Qu et al. 2014) and bioengineering (Waheed et al. 2016; Floren et al. 2016). Maintaining the 3D structures of the TE is pivotally depending on the ability of the hydrogel polymer to form homogeneous matrix when incorporated in an bioink. For instance, when hydrogel is used as a tissue scaffold (Billiet et al. 2012), internal pore structure and tissue vasculature should be mimicked to maintain cell viability (Ma et al. 2016; Liu and Bhatia 2002; El-Sherbiny and Yacoub 2013; Raman et al. 2016). Similarly, in micro- and nanofabrication, controlling hydrogel structure with high fidelity is so important to ensure success of resultant tissue viability (Ling et al. 2007; Ziaie et al. 2004; Slaughter et al. 2009). Hydrogels are highly hydrated 3D networks of cross-linked hydrophilic polymer chains and have been widely investigated for use in medical and TE tasks including bioactive delivery, cell carriers, TE scaffolds and wound healing. Hydrogels can be modified to alter their chemical, electrical, mechanical and thermal characteristics and can even be prepared to be electric conductors. Recent strategies in bioink have succeeded to incorporate carbon-based nanomaterials such as carbon nanotubes and graphene, clay nanomaterials, ceramics, fibres as well as growth factors to produce hybrid and huge types of composite hydrogels (Mobaraki et al. 2020). Controlling the mechanical and degradation properties of the hydrogel-based bioinks can be achieved by chemical functionalization, and/or increasing their response to stimuli is the keystone for the bioprinting process (Ji et al. 2020).

23.8 Bioinks

The bioinks are those bioplastic polymers based on cytocompatible hydrogel precursors that are compatible with different bioprinting approaches (Mobaraki et al. 2020). They are used to fabricate engineered or artificial living tissues and are often used with other additives to envelope living cells selected from human stem cells (Selcan 2018). These inks are considered as one of the most advanced tools for tissue engineering (TE) and regenerative medicine (Cui et al. 2020). They must have specific characteristics, including mechanical, rheological, biocompatibility and biofunctional features. The bioinks can be produced in mass production with high resolution and precise control for the fabricated bio-scaffold products (Selcan 2018). For instance, a hybrid bioink composed of alginate and cellulose nanocrystals (A-CNCs) was found to have excellent shear-thinning property and can be easily extruded through the nozzle providing proper initial shape fidelity (Wu et al. 2018). Developing proper hybrid bioinks is a valuable strategy that integrates the desirable characteristics of the constituents (Wu et al. 2018). As shown in Fig. 23.7, bioinks can be classified into four types, namely structural, sacrificial, functional and supportive. This classification has been driven by the role of the constituent materials in the final function of a printed construct rather than by the fabrication procedure itself.

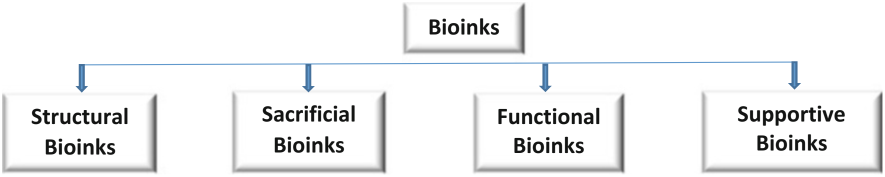

Fig. 23.7 Types of bioinks used for medical applications

23.8.1 The Structural Bioinks (S_tB)

Structural bioinks can be imagined as the ‘framework’ of the bioprinted scaffolds and are used to provide mechanical integrity to printed structures (Groll et al. 2019). Many precursors can be utilized under such category including alginates, chitosans, collagens, gelatin, hyaluronic acids and decellularized extracellular matrix (ECM). Important results can be achieved using them concerning mechanical and degradation behaviour, construct shape and size and cell viability.

23.8.2 The Sacrificial Bioinks (S_aB)

The S_aB permit introducing void volume within a bioprinted construct to create vascular networks during the bioprinting process. Increasing in the porosity of the bioprinted construct helps the nutrients added to feed the living stem cells to be diffused more easily. It is worth mentioning that this type of bioinks can be rapidly removed from the construct after making the desired permeability (Groll et al. 2019). The internal pore structure is the keystone for maintaining and enhancing cell viability. This type of bioinks is water-soluble under specific temperatures or can be rapidly degraded to help them to be isolated from the construct. They include non-cross-linked natural polymers including gelatin, pluronic acid and other materials that can be easily removed.

23.8.3 The Functional Bioinks (FBs)

The FBs are important to bioprinted constructs with incorporating the same functionality of the parent tissue or organ to be mimicked by providing biochemical, mechanical or electrical properties to influence cellular behaviour after a structure printing (Groll et al. 2019). The FBs possess specific compositions that are useful to control the cell differentiation and enhance their self-behaviour toward the final 3D-bioprinted product. For instance, these materials may include growth factors and other biological agents that are able to stimulate the stem cells to be differentiated into certain tissues. Furthermore, FBs may sequestrate and release growth factors or other molecules either added according to the scheme applied or released by the living cells incorporated within the bioink formula to recapitulate the native extracellular matrix (ECM). Other additives such as mineral microparticles or conductive molecules may be included into the FB formula to guide differentiation toward osteogenic or muscular tissue. Beyond the molecular cues, the FBs can provide different topographical cues to the cells to directly influence their organization and morphology.

23.8.4 The Supportive Bioinks (S_uB)

Bioprinted constructs may possess inadequate mechanical properties until complete curing of the tissue and need protection by the help of the S_uB, especially in vivo. These S_uBs act as structural supports that reinforce the target structure to be rigid. The rigid regions provided by support bioinks can resist any deformation caused by clamping necessary for the bioreactor system. Furthermore, bioinks can be classified into four categories (Fig. 23.8), namely natural hydrogel-based bioinks, synthetic bioinks, composite-based bioinks and cell-based bioinks. Each category has its own polymers that can be utilized successfully for the TE scaffolds.

Ji et al. (2020) reported two novel bioink platforms from either norbornene-functionalized carboxymethyl cellulose (norCMC) or carbic-functionalized CMC (cCMC). Both bioink formulations showed autogelation without UV light, which permit for adjusting the viscosity of the bioink. In addition, both formulations formed cell-laden hydrogels with the exposure to the UV light. The effect of the UV radiation was attributed to the photoreaction occured due to presence of the thiolene cross-linker. Three different human stem cells, namely human mesenchymal stem cells (hMSCs), NIH 3 T3 fibroblasts and human umbilical vein endothelial cells (HUVECs), were successfully bioprinted using their novel bioink formulations. Investigating Fig. 23.9 and Table 23.1 revealed that the most favourable commercial bioinks are cellulose nanofibril series, alginate series, collagen series, gelatin series and chitosan series (Table 23.2).

23.9 Bioprinting for Specific Human Tissue

23.9.1 Myocardial Bioprinting

3D- and 4D-myocardial bioprinting have been proved to be useful for modern heart era (Ong et al. 2018). For aortic heart valve, Maxson et al. (2019) evaluated the recellularization possibility of the aortic heart valve scaffold bioprinted using collagen hydrogel in high concentration incorporated with rat mesenchymal stem cells (MSCs). The suspension was 3D-printed into gelatin to produce disk scaffolds which were subsequently implanted subcutaneously in Sprague-Dawley rats for different durations, and the uniaxial tensile strength was characterized. Furthermore, the cell infiltration and inflammation were assessed via immunohistochemistry (IHC) and histological staining. Based on the stress-strain curves of the scaffolds, the scaffold transitioned through phases of resorption, synthesis, stabilization and ultimately

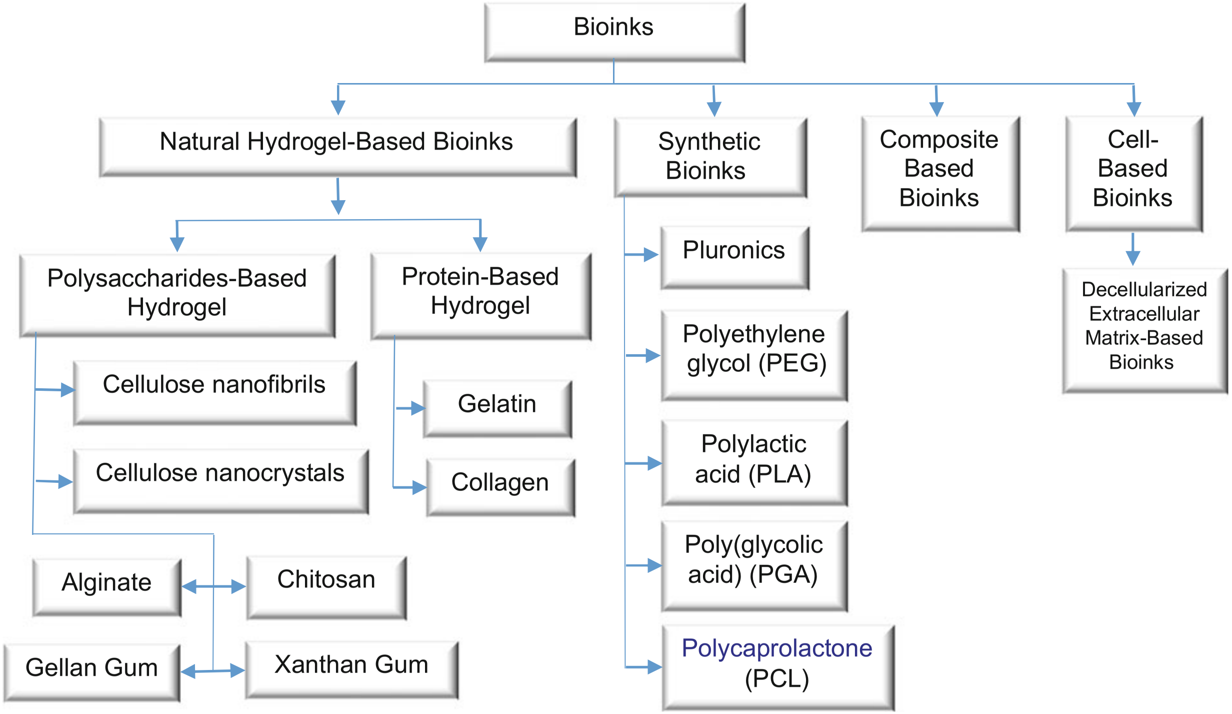

Fig. 23.8 Chemical classification of bioinks used for medical applications

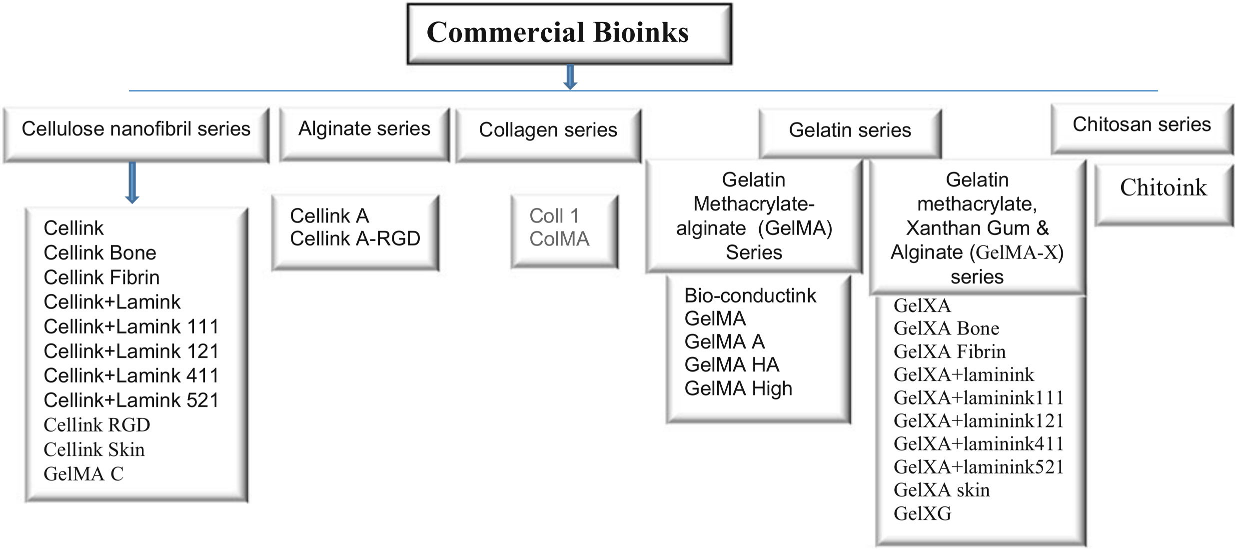

Fig. 23.9 Commercial series of bioinks used for medical applications

Table 23.1 Cellulose nanofibril-based bioinks

Commercial nanocellulose-based bioinks[a]	Composition	Cross-linkable	Applications
Cellink	Cellulose nanofibril + sodium alginate + [cross-linking agent {calcium chloride (0.57%) + D-mannitol + HEPES buffer solution}]	Ionic solution	Suitable for most cells including chondrocytes, bone, skin and mesenchymal stem cells. Useful for tumour engineering applications
Cellink bone	Cellulose nanofibril + sodium alginate + calcium phosphate tribasic + [cross-linking agent {50 mM calcium (0.57%)} + D-mannitol + HEPES buffer]	Ionic solution	Suitable for bone cells and mesenchymal stem cells. Useful for constructs with thermopolymers such as PCL and PLGA
Cellink fibrin	Cellulose nanofibril + sodium alginate + fibrinogen + [cross-linking agent {50 mM calcium (0.57%)} + D-mannitol + HEPES buffer]	Ionic solution + thrombin	Skin, endothelial, muscle and mesenchymal stem cells
Cellink + Laminink	Cellulose nanofibril + sodium alginate + laminink 111, 121, 411, 521 + [cross-linking agent {50 mM calcium (0.57%)} + D-mannitol + HEPES buffer]	Ionic solution	It is an excellent initiator for many cell types
Cellink Laminink 111	Cellulose nanofibril + sodium alginate + laminink 111 + [cross-linking agent {50 mM calcium (0.57%)} + D-mannitol + HEPES buffer]	Ionic solution	Suitable for brain, kidney, liver and intestinal cells. It has been used to study Parkinson's disease and cancer models
Cellink Laminink 121	Cellulose nanofibril + sodium alginate + laminink121 + [cross-linking agent {50 mM calcium (0.57%)} + D-mannitol + HEPES buffer]	Ionic solution	Suitable for skeletal muscle, brain, kidney and liver cells. It is widely expressed during embryonic and primary development of the lining of tissues and organs
Cellink Laminink 411	Cellulose nanofibril + sodium alginate + laminink 411 + [cross-linking agent {50 mM calcium (0.57%)} + D-mannitol + HEPES buffer]	Ionic solution	Suitable for pancreatic, vascular, immune, neural and hematopoietic stem cells. It can be used to study brain and nerve tissues
Cellink Laminink 521	Cellulose nanofibril + sodium alginate + laminink 521 + [cross-linking agent {50 mM calcium (0.57%)} + D-mannitol + HEPES buffer]	Ionic solution	Suitable for hepatic, pancreatic, vascular, neural, cardiac and undifferentiated stem cells. It is the natural laminin for pluripotent and embryonic stem cells

(continued)

Table 23.1 (continued)

Commercial nanocellulose-based bioinks[a]	Composition	Cross-linkable	Applications
Cellink RGD	Cellulose nanofibril + sodium alginate + synthetic peptide + [cross-linking agent {50 mM calcium (0.57%)} + D-mannitol + HEPES buffer]	Ionic solution	Suitable for skin, bone, muscle and mesenchymal stem cells
Cellink skin	Cellulose nanofibril + sodium alginate + [cross-linking agent {50 mM calcium (0.57%)} + D-mannitol + HEPES buffer]	Ionic solution + thrombin	Suitable for skin cells such as fibroblasts, keratinocytes and melanocytes
GelMA C	Cellulose nanofibril, gelatin methacrylate and alginate	365, 405 nm UV light + ionic solution	Suitable for most cells, such as skin, bone, muscle, neural and mesenchymal stem cells

[a] Based on the trade name registered by the producers

remodelling. This was confirmed by IHC and histology which showed favourable remodelling capacity indicating great tool for a 3D-printed heart valve.

23.9.2 Auricular Cartilage Tissue Engineering

Understanding the cardiac cellular composition and stating clear strategies to enhance vascularization and viability had forced this field to an upper advancement. In addition, in vivo studies utilizing small animal models have been challenged to get this target (Ong et al. 2018). The efficiency of auricular cartilage tissue engineering for repairing patients auricular defects was evaluated by using a bioink constituted of CNF and alginate (CNF-A) at a scale of either gene expression or protein levels. Relevant to an aqueous 3D environment, it was found that the CNF-A bioink enhances redifferentiation of the human nasal chondrocytes (HNCs) and neo-synthesis of cartilage-specific extracellular matrix components to offer good printability results. Furthermore, it facilitates the biofabrication of cell-laden, patient-specific auricular constructs with an open inner structure, high cell density and homogeneous cell distribution. The cell-laden CNF-A constructs gave an excellent result for the shape and the size stability as well as an increase in cell viability and proliferation during in vitro culture (Avila et al. 2016).

Avila et al. (2016) 3D-bioprinted auricular constructs laden with HNCs on gene expression as well as on protein levels. They cultured their experiment for up to 28 days, and the redifferentiation capacity of HNCs in CNF-A is studied. The target of auricular cartilage TE is to repair auricular defects. Bioprinting has the ability to control the placement of cells, biomaterials and biomolecules. Using CNF-A bioink

Table 23.2 Alginate-, collagen- and gel-based bioinks

Commercial bioinks[a]	Composition	Cross-linkable	Remark
Alginate series			
Cellink A	Alginate	Ionic solution	Suitable for cartilage, bone and mesenchymal stem cells. It can be used in cell differentiation, tissue formation and drug delivery
Cellink A-RGD	Alginate coupled with L-arginine-glycine-L-aspartic acid peptide	Ionic solution	It was optimized for enhanced cell adhesion which makes it useful for most cell types. It is commonly used with skin, muscle cells, iPSCs and mesenchymal stem cells. Its sequence has been used for improving integration of bone and vascular implants with the body
Collagen series			
Coll 1	Collagen I (5%) + acetic acid (0.12%)	Self-assembly	Suitable for most cells, including skin, bone, muscle, neural and mesenchymal stem cells
ColMA	Collagen methacrylate (5%) + acetic acid (0.12%)	Self-assembly, then 365, 405 nm UV light	Suitable for most cells, including skin, bone, muscle, neural and mesenchymal stem cells. It is thermo-reversible
Gelatin methacrylate (GelMA) series			
Bio Conductink	Gelatin methacrylate + water + single-walled carbon nanotubes + lithium phenyl-2,4,6-trimethylbenzoylphosphinate + HEPES buffer solution + Pluronic F-127	365, 405 nm UV light	Suitable for most cells. Designed for neural lineage, cardiac and skeletal muscle cells. Facilitates cellular communication through electrical potential variations
GelMA	Gelatin methacrylate	365, 405 nm UV light	Suitable for most cells, including skin, bone, muscle, neural and mesenchymal stem cells
GelMA A	Gelatin methacrylate and alginate	365, 405 nm UV light + ionic solution	Suitable for most cells, including skin, bone, muscle, neural and mesenchymal stem cells
GelMA HA	Gelatin methacrylate and methacrylated hyaluronic acid	365, 405 nm UV light	Suitable for skin, cartilage, bone, connective tissue, nerves and many other applications

(continued)

Table 23.2 (continued)

Commercial bioinks[a]	Composition	Cross-linkable	Remark
GelMA high	High-concentration gelatin methacrylate	–	Suitable for most cells, including skin, bone, muscle, neural and mesenchymal stem cells
Gelatin methacrylate-xanthan gum (GelMA-X) series			
GelXA	Gelatin methacrylate, xanthan gum and alginate	365, 405 nm UV light + ionic solution	Suitable for most cells, including skin, bone, muscle, neural and mesenchymal stem cells
GelXA bone	Gelatin methacrylate, xanthan gum, alginate, tricalcium phosphate and hydroxyapatite	365, 405 nm UV light + ionic solution	Suitable for bone cells and mesenchymal stem cells
GelXA fibrin	Gelatin methacrylate, xanthan gum, alginate and fibrinogen	365, 405 nm UV light + ionic solution + thrombin	Suitable for skin, endothelial, muscle and mesenchymal stem cells
GelXA + Laminink	Gelatin methacrylate, xanthan gum, alginate and laminin blend	365, 405 nm UV light + ionic solution	An excellent initiator bioink for many cell types
GelXA + Laminink 111	Gelatin methacrylate, xanthan gum, alginate and laminin a1b1y1[a]	365, 405 nm UV light + ionic solution	Suitable for brain, kidney, liver and intestinal cells as well as to study Parkinson's disease and cancer models
GelXA + Laminink 121	Gelatin methacrylate, xanthan gum, alginate and laminin a1b2y1[a]	365, 405 nm UV light + ionic solution	Suitable for skeletal muscle, brain, kidney and liver cells. It enhances study of organ and tissue lining in embryonic and early development phases
GelXA + Laminink 411	Gelatin methacrylate, xanthan gum, alginate and laminin a4b1y1[a]	365, 405 nm UV light + ionic solution	Suitable for pancreatic, vascular, immune, neural cells and hematopoietic cells
GelXA + Laminink 521	Gelatin methacrylate, xanthan gum, alginate and laminin a5b2y1[a]	365, 405 nm UV light + ionic solution	Suitable for hepatic, pancreatic, vascular, neural, cardiac and undifferentiated stem cells
GelXA skin	Gelatin methacrylate, xanthan gum, alginate and fibrinogen	365, 405 nm UV light + ionic solution	Suitable for skin cells (e.g. fibroblasts, keratinocytes and melanocytes)
GelXG	Gelatin methacrylate and xanthan gum	365, 405 nm UV light	Suitable for most cells, such as skin, bone, muscle, neural and mesenchymal stem cells

[a] Based on the trade name registered by the producers

is extremely assessed for auricular cartilage TE. Martínez et al. (2016) bioprinted auricular constructs laden with HNCs by culturing for up to 28 days, and the redifferentiation capacity of HNCs in CNF-A is studied on gene expression as well as on protein levels. It was found that this bioink facilitates the biofabrication of cell-laden, specific auricular constructs with an open inner structure, high cell density and homogeneous cell distribution supporting the redifferentiation of HNCs and neo-synthesis of cartilage-specific extracellular matrix components. This demonstrated that CNF-A bioink supports the redifferentiation of HNCs offering suitable printability in aqueous conditions, making it a promising technique for auricular cartilage TE and many other medical applications (Martínez et al. 2016). Anatomically shaped cartilage constructures, such as a human ear and sheep meniscus, were 3D-printed successfully using a CNF/alginate composite bioink (Markstedt et al. 2015).

23.9.3 Articular Cartilage of the Knee Joint

It is worth mentioning that the meniscus is able to distribute loads for protecting the articular cartilage of the knee joint from excessive stresses. Accordingly, harming the inner layers of their avascular region do not lead to healing, function disruption, while increasing the risk for knee osteoarthritis. Meniscus tissue engineering aims to restore normal meniscus function by the use of regenerated TE. Szojka et al. (2017) designed and characterized 3D-printed polycaprolactone scaffolds of the meniscus extracellular matrix with different fibre architectures and fibre spacing. The resultant scaffolds were produced using layer-by-layer technique to recapitulate both the shape and structural components of the native tissue and have proved to be a promising strategy for meniscus TE (Szojka et al. 2017).

23.9.4 Liver-Mimetic Honeycomb

The cellulose nanocrystals doped alginate (CNCs-A) bioink was utilized to bioprint a liver-honeycomb 3D structure including fibroblast and hepatoma cells. The biomatrices were cross-linked with $CaCl_2$ and was found to give minimal cell damage that makes it an attractive bioprinting material (Wu et al. 2018).

23.9.5 Bone Health and Regeneration

Bone health and regeneration of tissues and organs are crucial for human health in order to find better materials more suitable for this field. A novel formula of tricalcium phosphate (TCP), polycaprolactone (PCL) and bovine hydroxyapatite (BHA) was imparted to create scaffolds with fused deposition 3D-printing technique by Moukbil et al. (2020). They found that 15% (wt/wt) BHA fabricated composite scaffolds having more bioactivity than other cases with increasing proliferation and growth rates. 3D bioprinting is a quick developing technology and considered as a promising remedial therapy in orthopaedics. Recent studies have focused on cartilage and bone biology, standardization of cell culture schemes and protocols, bioink formulas and 3D-bioprinting technologies (Semba et al. 2020).

23.9.6 Osteochondral Tissue

Because of osteochondral tissue structure is complex over the full thickness spanning the subchondral bone region beneath the joint surface, advanced bioinks and the 3D bioprinters can offer the best solution to this problem (Nowicki et al. 2020). They designed an osteochondral tissue scaffold having multi-phasic layers by using polycaprolactone which is prepared from polycaprolactone-triol, castor oil and polyhexamethylene-diisocyanate precursors. They synthesized nanocrystalline hydroxyapatite (nHA) that was printed into the subchondral bone layers, while chondrogenic growth factors were introduced into the cartilage layer. Their results indicated that the 3D-printed scaffolds with nHA and bioactive cues have enhanced each of mechanical characteristics and hMSC adhesion, growth and differentiation (Nowicki et al. 2020).

23.9.7 Human Neuroblastoma

There are several factors in extrusion-based 3D-bioprinting field using different materials that needed to be fine-tuned to give the target print resolution as well as cell viability. To achieve this aim, a process termed as parameter optimization index (POI) was introduced. The POI determines and controls the optimal bioprinting conditions, especially printer speed and pressure affecting the bioink while it pass through the nozzles in order to achieve the highest printing accuracy and histological features resolution maintaining the theoretical shear forces at the lower level. Lewicki et al. (2019) applied the POI to optimize the process of 3D-bioprinting human neuroblastoma cell using a bioink hydrogel composed of sodium alginate using freeform reversible embedding of suspended hydrogels (FRESH). They found a significant difference between optimal parameters for printing SA (2%) with as well as without living cells in the bioink hydrogel. They also detected a notable effect of long-term cell culture on the printed scaffold. They obtained about 83% of viable cells during the first week of cell culturing using SA (2%) and FRESH bioprinting. These findings can be further performed to synthesis in vitro neuroblastoma 3D-culture systems for cancer research (Lewicki et al. 2019).

23.9.8 Cancer Tumour

Cancer is one of the most serious diseases threaten the humanity. The 2D-cancer models have some limitations such as insufficient biomimicry of the heterogeneous conditions of tumours and their 3D structures. Contradiction between the experimental models and those for actual tumour greatly impairs understanding the carcinogenesis process and development of effective remedies against cancer. On the other hand, 3D modelling of cancer tumour bioprinting have great potential to solve these problems (Oztan et al. 2020).

23.9.9 Fat Grafting

Autologous fat grafting has been applied for correcting soft-tissue distortions. 3D-bioprinting techniques enable making vast architectures retaining their parent vascular patterns. Recently, adipose tissue-derived stromal/stem cells (ASCs) have been used for graft survival successfully. Saljo et al. (2020) obtained high viability and good printability of human lipoaspirate containing ASC in alginate/nanocellulose bioink and vascular structures was preserved using the 3D-bioprinted grafts. Furthermore, it was indicated that novel blood vessels were appeared on the graft surface after 30 days in vivo confirming the occurrence of angiogenesis into the graft, as well as vascularization in the centre of the tissue. In addition, histologic and immunohistochemical examinations confirmed the presence of potential ASCs upon the first week in vivo (Saljo et al. 2020).

23.10 Computed Tomography (CT) Scan and Hounsfield Unit (HU)

A CT scan is a computerized X-ray imaging tool to produce tomographic images that permits a body to be visible without cutting. Cone beam computed tomography (CBCT) is one of the techniques in which the X-rays are divergent, forming a cone-shaped beam. It is used for many clinical applications, due to its low cost, easy to apply and low radiation emission compared with multi-slice computerized tomography (De Vos et al. 2009). In addition, the Hounsfield scale is known to be a quantitative scale to define radiodensity. It is frequently used in CT scans to estimate its CT number. There is a great attention in applying the CT and Hounsfield unit (HU) measurements for either identification or assessment of non-calcified plaque despite of the resultant complex geometry of the coronary arteries that prevents to obtain good image quality. In order to investigate coronary artery disease carefully, cardiac motion must be considered to enhance accurate diagnoses (Stepniak et al. 2020). Since image quality and Hounsfield unit measurements of the arterial network vary and depend on different factors, Stepniak et al. (2020) have suggested plaque phantom design to study the effect of non-calcified plaque geometry and arterial motion on different factors as a CT-optimization study. In their investigation, they have demonstrated the manufacturing process of a plaque phantom of the coronary considering the accurate physiological geometry. They obtained a computer model by segmentation of CTCA images using various materials to 3D printing of the model. The static and dynamic mechanical properties of the 3D-printing product are characterized, and the CT numbers of contrast-enhanced tubular specimens with 50%, 75% and 90% concentric stenosis are determined and compared to the ranges for lipid-rich and fibrous plaque.

23.11 Conclusion

The bioprinting technology depends essentially on stem cells incorporated in bioplastic polymers termed as bioink and growth factors. The stem cells are essential for the TE due to their ability to differentiate into several types of functionally different cells according to the stimuli affecting them. Bioinks may be prepared from natural or synthetic single biomaterial or a homogenized hybrid material. An ideal bioink must have suitable mechanical, rheological and biological characteristics that are essential to adapt the parent functionality of the target tissues. Performing bioprinting technology, a bioink solution containing desired living cells is allowed to be cross-linked and cured immediately to regenerate the desired tissue model. The bioprinting technology has been used for mimicking specific human tissues, including myocardial treatments, auricular cartilage, articular cartilage of the knee joint, liver-mimetic honeycomb, bone health and regeneration, osteochondral tissue, human neuroblastoma, cancer tumour and fat grafting. The computed tomography and Hounsfield unit measurements have gained more attention for either identification or assessment of non-calcified plaque.

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