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Polyhydroxyalkanoates

Biosynthesis, Chemical Structures and Applications

Harvey Williams ▪ Patricia Kelly
Editors

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PREFACE

Polyhydroxyalkanoates: Biosynthesis, Chemical Structures and Applications opens with an exposé on employing extremophiles as polyhydroxyalkanoate (PHA) producers. The authors suggest that extremophiles may be easily subjected to a long-term continuous cultivation processes, which considerably enhances overall productivity while reducing the energy demand in biopolymer production. Conversely, a range of challenges remain, including improving the metabolic capability of extremophiles, recycling of fermentation broth, various process engineering aspects, and adaptation of bioreactor materials and process controlling devices to conditions shortening their life span. Following this, the enzymes, regulators and genes involved in PHA biosynthesis are analyzed for their potential as an alternative to synthetic polymers. They are synthesized as intracellular carbon and energy storage compounds from over 300 species in the presence of excess carbon and under oxygen, nitrogen or phosphorus limitation, or after pH shifts. This collection goes on to suggest PHA as a promising alternative for petrochemical compounds. The challenges of increasing economic feasibility in the global market, minimizing costs, and improving the polymer yield are reviewed. Additionally, recent research on structural variations of PHAs has been centered on the design, biosynthesis, and properties of biodegradable and biocompatible materials, which can be used for bioengineering. This collection also includes a focus on the roles of polyhydroxyesters and PHAs in the construction of tissue engineering scaffolds, which are used in bone, cartilage, ligament, skin, vascular tissues, neural tissues and skeletal muscles. Their exceptional properties, such as high surface-to-volume ratio, high porosity with very small pore size, and biodegradation have made them gain a lot of attention in this field. The biomedical applications of PHAs are explored, including *in vivo* implants, tissue engineering, anticancer agents, drug delivery, biocontrol agents and memory enhancers, as their low acidity allows for minimal risk in usage. In order to enhance its applicability in various fields, the blends and nanocomposites of PHAs are studied and their potential challenges, applications and opportunities are addressed. After which, the industrial and

agricultural applications are described, with specific focus on potential applications of PHAs in packaging. Other applications include moulded goods, paper coatings, non-woven fabrics, adhesives, films and performance additives. Recent advances in this area, by means of peer-reviewed literature and patents, are introduced and discussed. Moreover, innovative strategies for the synthesis of novel polymer blends, adequate for food contact applications, are presented.

Chapter 1 - Various extremophilic microbial species originating from diverse extreme environments such as hot springs, biotrickling filters, salterns, glaciers, or heavily polluted habitats, are reported in the current literature to display more or less pronounced potential for polyhydroxyalkanoate (PHA) biopolyester biosynthesis. In context with the current quest for new strategies towards enhanced PHA production processes, the chapter summarizes the present state of research on PHA production under such extreme conditions of temperature, salinity, pH-value, or levels of precarious compounds, which, for most mesophilic PHA-accumulating species, are strongly growth inhibiting or even life threatening. The chapter exposes that employing extremophiles as PHA producers displays a double-edged sword: On the one hand, extremophiles can easily be subjected towards long-term continuous cultivation processes, which considerably enhances overall productivity of the process and also reduces the energy demand in biopolymer production regarding sterility precautions, cooling, or heating; this advantage paves the way towards more cost-efficient PHA production. As a further benefit, many extremophilic PHA producers turned out to utilize industrial waste streams as 2nd-generation feedstocks for PHA production, which not only safes feedstock costs, but, moreover, contributes to value-added, safe treatment of waste materials. Furthermore, in some cases, applying extremophiles even facilitates downstream processing for PHA recovery by exposing PHA-rich cells to conditions favoring their disintegration. On the other hand, one has to get in grips with a range of remaining challenges, such as improving the metabolic capability of extremophiles, recycling of precarious spent fermentation broth, various process engineering aspects, and adaptation of bioreactor materials and process controlling devices to conditions shortening their life span. A considerable number of promising reports on novel PHA production processes based on phylogenetically highly versatile extremophilic organisms is summarized and comparatively assessed in the chapter at hand. It is shown that some processes were already established under controlled bioreactor conditions, using both continuous and discontinuous cultivation regimes. When analyzing the products obtained by these processes, it becomes clear that PHA from extremophiles can easily compete with PHA from usually applied mesophilic biopolymer production strains in terms of material properties. In some cases, "extremophile PHA" even outperforms the processibility of PHA from well-established production processes, e.g., by displaying extraordinarily high molecular masses. Based on the available data, one can currently conclude that especially those extremophile PHA production processes using robust, extremophile organisms,

copiously accessible raw materials, and continuous cultivation mode hold realistic promise for future industrial-scale realization. Nevertheless, the route towards routinely implementing extremophile PHA producers is still cumbersome. Further assessment of novel and underexplored extremophile production strains with superior kinetics for growth and PHA accumulation on a range of inexpensive carbonaceous substrates will be needed in order to fully profit from the natural wealth of extremophile biopolyester producers. As suggested by individual studies, metabolic bottlenecks can be coped with genetic engineering in order to boost the kinetic performance of extremophiles. Considering the fact that the scientific community already has a rather clear picture about feasible inexpensive feedstocks to be used for cost-efficient PHA production, further process optimization has to emphasis on enhanced productivity and energy efficiency. Here, the application of robust extremophiles provides the chance to minimize energy for sterilizing, cooling, or heating.

Chapter 2 - Polyhydroxyalkanoates (PHAs), a biodegradable and biocompatible class of biopolymers, gained an increased interest nowadays as potential alternatives to synthetic polymers. They are synthesized as intracellular carbon and energy storage compounds from over 300 species, mainly bacteria, in the presence of excess carbon and under oxygen, nitrogen or phosphorus limitation, or after pH shifts. Although most bacteria accumulate PHAs under stress conditions, there are some of them that do not require nutrient limitation for PHA synthesis such as *Alcaligenes latus*, or recombinant *E. coli*. In the first case, PHAs are degraded and used for bacterial growth when a limiting nutrient is provided. Therefore, bacteria that produce PHAs have both biosynthetic and degrading enzymes. For PHAs biosynthesis three pathways have been elucidated so far. In pathway I, 3HB monomers are generated by the condensation of two acetyl-CoA molecules from the tricarboxylic acid (TCA) cycle. In the second pathway (II) the substrates are generated by β-oxidation of fatty acids, while in pathway III monomers are generated from structurally unrelated and simple carbon sources such as glucose, sucrose and fructose. Gene's organization of the corresponding biosynthetic enzymes also differs in PHAs producing bacteria. The key enzyme is PHA synthase and significant effort has been made to generate synthases with enhanced activity and substrate specificity. More than 150 monomers have been reported so far; their composition influences the physical properties and characteristics of the polymer, thus particular notice is given to the carbon source that will be used. Recombinant strains also provide an alternative way to produce PHAs with novel properties from inexpensive raw materials. The plethora of enzymes, regulators as well as their genes involved in PHAs biosynthesis will be discussed in details.

Chapter 3 - Polyhydroxyalkanoates (PHAs) are bacterial polyesters belonging to the most important group of bio-based and biodegradable polymers. A large number of bacteria possesses the ability to synthesize PHAs as carbon energy storage and a variety of monomer constituents of PHAs has been described, whose chemical composition

range from straight to aromatic structures. The PHA synthesis is strongly influenced by the carbon source utilized for microbial growth and the metabolic pathways involved in the polymer synthesis, which the PHA synthase plays an essential role in the PHA polymerization process. This chapter introduces PHAs from their biochemical concept to a promising alternative for petrochemical compounds as eco-friendly thermoplastics. Among many suitable characteristics for industrial applications the most important attribute of PHAs is their biodegradability. The challenges faced by the industry of biopolymers in order to scaling-up the PHA production and increase their economical feasibility in the global market, including recent advances to minimize costs and to improve the polymer yield, besides the sustainable production of PHAs from agro-industrial by-products are also a focus of this review chapter.

Chapter 4 - One of the well-known biopolymer groups generated by direct biosynthesis from renewable resources is the family of Polyhydroxyalkanoates (PHAs). It is a representative category of structurally diverse intracellular biopolyesters accumulated by many bacteria as carbon and energy storage granules. PHA molecules are typically constituted by numerous (R)-hydroxy-fatty acid monomer units. Each monomer unit bears a side chain R group, which is usually either saturated or unsaturated alkyl groups, branched alkyl groups, and substituted alkyl groups, although these forms are less common. PHAs are homo-, co- and terpolymers, generally classified into short-chain-length PHAs (*scl*-PHAs) and medium-chain-length PHAs (*mcl*-PHAs) by the different number of carbons in their repeating units. The main polymer of the PHA family is the homopolymer Polyhydroxybutyrate P(3HB). The number of differences in PHA properties profile depends on the: i) variety of monomers, ii) constitutional isomerism, iii) wide range of molecular weights, and iv) physical and/or chemical modifications of their microstructures. Recent research on structural variations of PHAs has been directed towards the design, biosynthesis, and properties of biodegradable and biocompatible materials, which can be used for bioengineering of new optical and other smart chiral intermediates. Moreover, PHAs are exploited in a series of applications in the packaging and food industry, agriculture, medicine, pharmacy, and also as raw materials for synthetic chemistry, representing an interesting source for smaller molecules or chemicals. Therefore, due to their commercial interests, PHAs are a promising group of materials for future study related to synthetic mechanisms, monomer diversity, physiological roles, and controllable production.

Chapter 5 - Tissue engineering is a field that has gained a lot of advancement since the discovery of biopolymers. Biopolymers are simply polymers that are made-up of polymeric biomolecules. They consist of monomeric units that are covalently bonded to one another in order to form very large structures. Biopolymers have been widely used as biomaterials for the construction of tissue engineering scaffold. Scaffolds have been used for tissue engineering such as bone, cartilage, ligament, skin, vascular tissues, neural tissues and skeletal muscles. Polyhydroxyesters are typical examples of synthetic

biopolymers that have been employed for this application. Their exceptional properties, such as: high surface-to-volume ratio, high porosity with very small pore size, biodegradation, and mechanical property have made them gain a lot of attention in this field. Also, they have advantages which are significant for tissue engineering. This chapter focuses on polyhydroxyesters, such as: PLA (Polylactide), PGA (Polyglycolide or poly(glycolic acid)), PCL (Polycaprolactone) and PLGA (Poly(lactide-*co*-glycolide), which have diverse applications in tissue engineering. Details of these polyhydroxyesters and their application in tissue engineering will be discussed in this chapter.

Chapter 6 - Tissue engineering is a field that has gained a lot of advancement since the discovery of biopolymers. Biopolymers are polymers produced by living organisms; that is, they are polymeric biomolecules. They consist of monomeric units that are covalently bonded to one another in order to form larger structures. Biopolymers have been widely used as biomaterials for the construction of tissue engineering scaffold. Scaffolds have been used for tissue engineering, such as: bone, cartilage, ligament, skin, vascular tissues, neural tissues, and skeletal muscles. Polyhydroxyester is a typical example of biopolymers that have been employed for this application. Their exceptional properties such as high surface-to-volume ratio, high porosity with very small pore size, biodegradation, and mechanical property have made them gain a lot of attention in this field. Also, they have advantages which are significant for tissue engineering. This chapter will focus on the production, modification, properties and medical applications of polyhydroxyesters, such as PLA (Polylactide), PGA (Polyglycolide or poly(glycolic acid)), PCL (Polycaprolactone), poly(ester amide)s and PLGA (Poly(lactide-co-glycolide), with particular emphasis on the different polyhydroxyalkanoates (PHAs), which have diverse applications in tissue engineering.

Chapter 7 - There has been a growing interest in the replacement of fossil-based polymers with biodegradable polymers, especially those produced through natural resources. Among these biodegradable polymers, polyhydroxyalkanoates with their unique properties, e.g., high molecular weight (similar to conventional polymers), qualify them to be promising candidates for various industrial applications. Their high cost and their relatively low mechanical properties, limit their applications. Blending and the incorporation of various fillers, have been studied for the past decades in order to improve and/or overcome these drawbacks. In this chapter, recent studies, based on the blends and nanocomposites of polyhydroxyalkanoates in order to enhance its applicability in various fields, are reviewed. The potential applications, challenges and opportunities relating to polyhydroxyalkanoates blends and their nanocomposites, are also addressed.

Chapter 8 - Polyhydroxyalkanoates (PHAs) are becoming very popular in the biodegradable polymer market. This is because of their promising properties, such as high level biodegradability in different environments. Amongst the well-known biopolymers, these biogenic polyesters (Figure 1) (PHAs) emerge as potential suitable

and sustainable replacements for fossil fuel-based thermoplastics. PHA can be produced from bacteria cell and then formulated and processed by extrusion for the production of rigid and flexible plastics. The applications of PHA, include: packaging, moulded goods, paper coatings, non-woven fabrics, adhesives, films and performance additives. The present chapter reviews the different classes and applications of PHAs, which include: industrial, agricultural, with specific focus on potential applications of PHAs in packaging.

Chapter 9 - The ideal polymeric material for direct food contact applications should possess specific characteristics and properties. These properties include the preservative role of the packaging materials against external mechanical damages and bacterial spoilage of the contained food. They must possess the ability to act as gas and liquid barriers providing protection against oxidation and moisture. These types of materials should also be non-toxic, versatile, and compatible with the majority of the food, biodegradable, sustainable, practicable, and easy to be formed through common polymer morphing processes. In this direction, polyhydroxyalkanoates (PHAs) biopolyesters are probably the only group of polymeric materials that fulfill all the aforementioned criteria. PHAs are biocompatible and biodegradable, while they exhibit enhanced barrier properties. These biopolymers are also compatible with various materials, such as other polymers, organic or inorganic nanoparticles, antimicrobial agents, etc. and consequently, they can be extensively modified towards the synthesis of novel smart, active and functional food packaging materials. This chapter is focused on PHA applications in food packaging and food contact. Recent advances in this area, by means of peer-reviewed literature and patents, are going to be introduced and discussed. Additionally, innovative strategies towards the synthesis of novel polymer blends, adequate for food contact applications, will be presented. Moreover, all the recent technologies and processes for the synthesis of functional surfaces based on functional PHAs, nanocomposites of PHAs and blends of PHAs with other polymers will also be reported.

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Chapter 1

PHYSIOLOGICAL, KINETIC, AND PROCESS ENGINEERING ASPECTS OF POLYHYDROXYALKANOATE BIOSYNTHESIS BY EXTREMOPHILES

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ABSTRACT

Various extremophilic microbial species originating from diverse extreme environments such as hot springs, biotrickling filters, salterns, glaciers, or heavily polluted habitats, are reported in the current literature to display more or less pronounced potential for polyhydroxyalkanoate (PHA) biopolyester biosynthesis. In context with the current quest for new strategies towards enhanced PHA production processes, the chapter

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summarizes the present state of research on PHA production under such extreme conditions of temperature, salinity, pH-value, or levels of precarious compounds, which, for most mesophilic PHA-accumulating species, are strongly growth inhibiting or even life threatening.

The chapter exposes that employing extremophiles as PHA producers displays a double-edged sword: On the one hand, extremophiles can easily be subjected towards long-term continuous cultivation processes, which considerably enhances overall productivity of the process and also reduces the energy demand in biopolymer production regarding sterility precautions, cooling, or heating; this advantage paves the way towards more cost-efficient PHA production. As a further benefit, many extremophilic PHA producers turned out to utilize industrial waste streams as 2nd-generation feedstocks for PHA production, which not only safes feedstock costs, but, moreover, contributes to value-added, safe treatment of waste materials. Furthermore, in some cases, applying extremophiles even facilitates downstream processing for PHA recovery by exposing PHA-rich cells to conditions favoring their disintegration. On the other hand, one has to get in grips with a range of remaining challenges, such as improving the metabolic capability of extremophiles, recycling of precarious spent fermentation broth, various process engineering aspects, and adaptation of bioreactor materials and process controlling devices to conditions shortening their life span.

A considerable number of promising reports on novel PHA production processes based on phylogenetically highly versatile extremophilic organisms is summarized and comparatively assessed in the chapter at hand. It is shown that some processes were already established under controlled bioreactor conditions, using both continuous and discontinuous cultivation regimes. When analyzing the products obtained by these processes, it becomes clear that PHA from extremophiles can easily compete with PHA from usually applied mesophilic biopolymer production strains in terms of material properties. In some cases, "extremophile PHA" even outperforms the processibility of PHA from well-established production processes, e.g., by displaying extraordinarily high molecular masses.

Based on the available data, one can currently conclude that especially those extremophile PHA production processes using robust, extremophile organisms, copiously accessible raw materials, and continuous cultivation mode hold realistic promise for future industrial-scale realization. Nevertheless, the route towards routinely implementing extremophile PHA producers is still cumbersome. Further assessment of novel and underexplored extremophile production strains with superior kinetics for growth and PHA accumulation on a range of inexpensive carbonaceous substrates will be needed in order to fully profit from the natural wealth of extremophile biopolyester producers. As suggested by individual studies, metabolic bottlenecks can be coped with genetic engineering in order to boost the kinetic performance of extremophiles.

Considering the fact that the scientific community already has a rather clear picture about feasible inexpensive feedstocks to be used for cost-efficient PHA production, further process optimization has to emphasis on enhanced productivity and energy efficiency. Here, the application of robust extremophiles provides the chance to minimize energy for sterilizing, cooling, or heating.

Keywords: alkaliphiles, Archaea, bacteria, biopolymers, extremophiles, haloarchaea, halophiles, metalophiles, polyhydroxyalkanoates (PHA), psychrophiles, stress factors, thermophiles

INTRODUCTION

Today we are observing countless efforts globally dedicated to make processes of "white biotechnology" more and more efficient from an economic perspective. In this context, the scientific community and industrialists discuss the biomediated formation of different mass and niche products based on the transformation of renewable raw materials by living organisms, or by using their biocatalytically active constituents such as isolated enzymes. Biosynthesis of several important products, both generated by the primary or the secondary metabolism of production strains is considered state-of-the-art; well-known compounds like acetic acid, bacterial and fungal antibiotics, carbohydrates, citric acid, ethanol, lactic acid, and many more are prime examples in this regard (Hermann and Patel 2007). In contrast to these examples, biosynthesized products with plastic-like properties, often referred to as so called "bioplastics" or "green plastics", are still in anticipation of their ultimate market penetration (Iles and Martin 213).

Among all "bioplastics", polyhydroxyalkanoates (PHA) are generally regarded as one of the most auspicious families. PHA are biosynthesized as polyoxoesters of hydroxyalkanoic acids by a steadily increasing number of eubacteria and representatives of the Archaea domain, hence, they are typical prokaryotic products (Tan et al. 2014). Figure 1 shows the general chemical structure of PHA, schematically embedded in a fictive prokaryotic cell. Microbiologically, these biopolymers serve as inert intracellular carbon- and energy reserves, which, in addition to their role as storage compounds to be metabolized during starvation, provide the cells with a survival benefit when exposed to various unfavorable environmental situations, such as desiccation, exposition to heavy metals, high salinity, organic solvents, oxidants, or radiation (Obruca et al. 2010a, Kourmentza et al. 2017a). When observed by a microscope, PHA inclusions, also referred to as "carbonosomes" due to their complex functional character, appear as bright, light-refractive granules (Jendrossek 2009). As a function of the hydroxyalkanoic acid monomers they are composed of, their material properties resemble those of well-established thermoplastics and elastomers stemming from petro-chemistry. Most importantly, PHA are currently considered the only family of materials with plastic-like features, which are embedded into a completely biological life cycle. This can be visualized by the following features (reviewed by Koller et al. 2010, 2017):

i. Their production is entirely based on the utilization of renewable feedstocks, such as carbohydrates (sugars and polysaccharides), lipids (oils, tallow, or fatty acid esters), alcohols (glycerol, methanol, etc.), or gaseous C1-compounds (CH_4, CO_2, etc.)
ii. Both biosynthesis of hydroxyalkanoic acids as the monomeric PHA building blocks and the subsequent polymerization of hydroxyalkanoic to PHA takes place in living prokaryotic cells by the strain's proteome, hence, it's enzymes.

This is in clear contrast to other so called "bioplastics" such as poly(lactic acid), where polymerization of the biologically produced monomers (lactic acid) occurs chemically, or to compostable "bioplastics" which consist of petrochemically produced monomers, such as the product Ecoflex™, which, although compostable, consists of petrochemically produced building blocks.

iii. As an outstanding feature, PHA´s monomers and oligomers are naturally present in the human body, therefore PHA display extraordinary biocompatibility, which enables *in vivo* application of these biopolyesters, thus their use in the medical field. In this context, PHA was successfully used in the past to produce biodegradable implants, pins, fibers, sutures, or meshes, scaffolds for repair of cartilage or nerves, and for many additional medical purposes.

iv. When PHA are biodegraded, they are completely decomposed by biological activity present in particular environments without formation of any hazardous compounds or particles; this is unlike many synthetic biodegradable polyesters. Merely biomass, water, and CO_2 result as the final products of PHA's aerobic breakdown; anaerobic decomposition, such as in biogas plants, results in formation of CH_4.

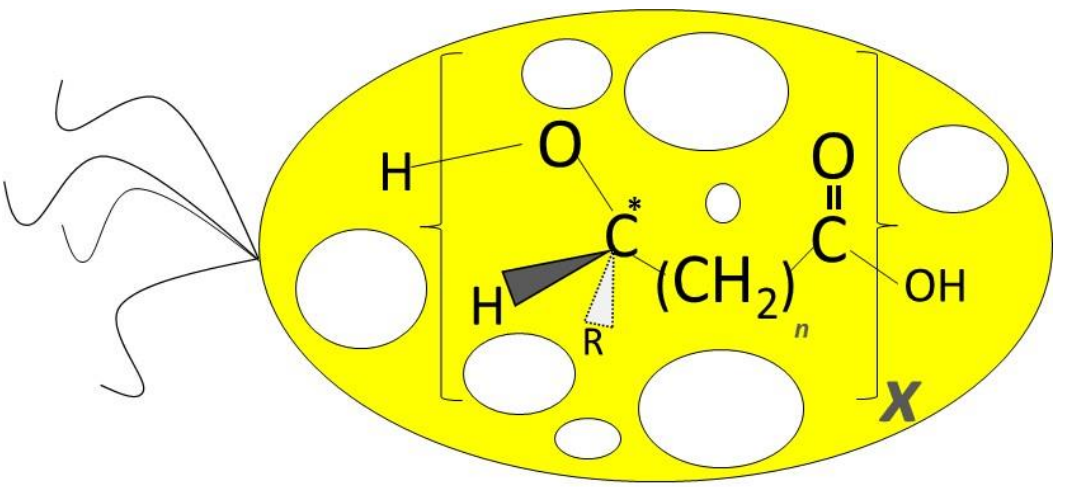

Figure 1. General chemical structure of PHA. White ellipses in the schematic prokaryotic cell represent PHA granules. **R**: side group of monomers (hydroxyalkanoic acids), **n:** number of methylene group in monomer ("backbone" of the monomer), **x**: degree of polymerization (number of monomers in PHA-polyester molecule). *: indicates the chiral center in most PHA building blocks (exception: the achiral building block 4-hydroxybutyrate).

A growing number of recent scientific studies evidences the environmental benefit of PHA over their competitors from petrochemistry, which typically display full-carbon-backbone polymers highly recalcitrant towards microbial degradation. These studies are frequently based on Life Cycle Assessment, which resorts to tools like the Sustainable

Process Index (Harding et al. 2007, Koller et al. 2013a, Narodoslawsky et al. 2015, Piemonte and Gironi 2011, Pietrini et al. 2007, Shahzad et al. 2013, Shahzad et al. 2017, Yates et al. 2013). However, environmental superiority is not sufficient to compete on the plastic market. Besides the "green" labeling, new polymeric materials must also reach the benchmarks of requested product quality, and PHA production needs to become competitive also in terms of production costs.

Product quality can be tailored by producing PHA co-, ter-, and quadropolymers of blocky structure or random distribution of building blocks, by triggering the molecular mass of PHA and its distribution, by post-synthetic chemical modification, and, to an emerging extend, by processing PHA together with compatible materials to blends and composites (Koller et al. 2017, Kourmentza et al. 2017). Especially the production of PHA-based composite materials is a topic of currently strongly increasing interest all over the world (Galego et al. 2000); in this context, numerous biological surplus materials such as (ligno)cellulosic fibers, nanocellulose whiskers, lignin, etc., can be used to design composites of tailor-made properties, e.g., increased biodegradation rate, or decreased density (Chiellini et al. 2004, Dasan et al. 2017; Khosravi-Darani and Bucci 2015, Kovalcik et al. 2015, Perez-Amaro et al. 2015). In addition to biological materials, also inorganic matter such as (nano)clays, borone nitride, etc., find application to provide the final composite material with enhanced properties in terms of gas permeability, processibility, or others (Bordes et al. 2009; Öner et al., 2017).

In order to increase economics of PHA production, a number of steps in this multi-facetted process have to be optimized, which needs the synergistic cooperation of experts from diverse scientific fields, such as microbiology, genetic engineering, process engineering, analytical chemistry and physical chemistry. In this context, major efforts are dedicated to assess novel, inexpensive carbon-rich materials, which can serve as feedstocks for PHA production. Well-established, expensive raw materials typically used in industrial biotechnology, such as saccharides or oils of nutritional value shall be replaced by inexpensive alternatives not competing human food chain in order to preserve food resources and to overcome industrial waste disposal problems (Jiang et al. 2016). A considerable number of different industrial surplus materials, mainly stemming from agriculture and the food industry, was already tested as feedstocks for PHA production. As examples, literature teaches us about the implementation of whey from dairy and cheese making industry (Koller et al. 2007a,b, Pais et al. 2016), residues of the biodiesel production and the animal processing industry (Muhr et al. 2013a,b, Titz et al. 2012, Hermann-Krauss et al. 2013), inexpensive and waste lipids (Kourmentza et al. 2017b, Walsh et al. 2015), or various amply available lignocellulosics (Obruca et al. 2015), such as fruit peels or hydrolyzed bagasse (Kulkarni et al. 2015), straw (Ahn et al. 2016, Cesário et al. 2014), wood dust (Kucera et al. 2017), paper industry rejects (Jiang et al. 2012), waste office paper (Annamalai et al. 2018), or even spent coffee ground from

gastronomy (Obruca et al. 2014). Beside the raw materials, it is important to develop novel processes to release PHA as intracellular products from microbial biomass; here, different strategies are currently investigated, most of them devoted to the reduction of the input of solvents, chemicals and energy into the PHA-recovery process. Such new recovery techniques are also intending to preserve the native properties of PHA, hence, its flexible quasi-amorphous state and its high molecular mass. In this context, green solvents, supercritical solvents, or, most recently, ionic liquids are used to extract PHA, in addition to new methods to easily disintegrate the microbial cell wall and various mechanical methods for cell disintegration (reviewed by Jacquel et al. 2009, Koller et al. 2013b, Madkour et al. 2013). Moreover, even the digestion of non-PHA biomass by insects and the subsequent excretion of intact PHA granules, which are less digestible by the animals, was reported (Murugan et al. 2016, Ong et al. 2017). Further, new process engineering approaches are in status of development, which aim at increasing biopolymer productivity and at the optimization of PHA's monomeric composition; in this realm, one-, two, and multistage continuous processes, based on chemo-, pH-, or turbidistat feeding regimes were designed in the recent years. These processes are designed in such a way to match the kinetics of PHA biosynthesis (first microbial growth phase under nutritionally balanced conditions, which is followed by a PHA-accumulation phase initiated by nutritional stress conditions, hence limitation of growth-essential medium components together with excess availability of exogenous carbon source) (reviewed by Kaur and Roy 2015, Koller and Braunegg 2015, Koller and Muhr 2014).

As a well-known problem in many biotechnological processes such as PHA production, contamination by alien microbes displays a major threat when carrying out bioreactor cultivations; this is especially true in the case of large-scale processes, where microbial contamination can endanger whole cultivation setups and therefore is risky for the economic viability of a new process in status of development (Pittijärvi et al. 1998). To overcome this problem, extremophile production strains can be used, which are best cultivated under such extreme conditions, which hinder the thriving of most microbial competitors (reviewed by Koller 2017). In many cases, this strategy even allows the cultivation under significantly reduced sterility precautions, or even without any sterilization of bioreactor equipment and nutritional medium (Yin et al. 2015). In the case of *Haloferax mediterranei*, the best-described extremophile (halophile) PHA-producer or the thermophile bacterium *Chelatococcus* sp. (Ibrahim and Steinbüchel 2010), cultivation setups were run for extended time periods without sterilizing the bioreactors; no microbial infection was noticed, hence, the cultures stayed monoseptic. Staying with the example of *Chelatococcus* sp. and considering the high amount of thermal energy generated by the cell's metabolism, a phenomenon that is especially effective in high cell density cultivations, such thermophilic cultivation processes can be operated as energy saving "self-heated" system. In addition, the input of heat caused by the stirring system of

the bioreactor adds to the heating of the cultivation broth. Moreover, cultivation processes using thermophilic production strains are also energetically efficient due to lower requirements on energetically demanding cooling system. This exemplifies that costs for both heating and cooling can be saved and, in analogy to the application of halophiles mentioned above, sterilization might not be needed when cultivating thermophilic microbes (Ibrahim and Steinbüchel 2010). Moreover, applying production strains that grow well under pH-conditions far from the pH-optimum of potential microbial contaminants also minimizes the risk of infection; as a well-known example, the application of alkaliphile production strains circumvents the growth of fungal contaminants in the cultivation setup (Gomes and Steiner 2004). Together with their significance to produce valued biotechnological products, alkaliphile organisms are also implemented in processes for bioremediation, such as neutralization of alkaline wastewater bodies, or removal of heavy metals (Mamo and Mattiasson 2016).

The current scientific literature exhaustively describes the potential of both microbial wild-type strains and of genetically engineered uni- and multicellular strains for PHA production. Both wild-type and engineered prokaryotes and engineered eukaryotes, such as yeasts, plants, insects, etc., evidence PHA accumulation (reviews by Koller et al. 2010, Koller et al. 2017, Kourmentza et al. 2017, Tan et al. 2014). Microbes are characterized by different optimum environmental cultivation conditions. In this context, biotechnology typically resorts to such production strains, which thrive best in the mesophilic range, i.e., at optima for the pH-value near the neutral range around 7.0, at moderate salt levels, and at temperature ranges of about 25 to 37°C. In contrast to such mesophiles, extremophile organisms flourish best under conditions that severely jeopardize or complete inhibit growth of most other organisms. In many cases, extremophiles even are not able to endure mesophile environments at all. Such extreme environments cover low or high temperature (–2 to 20°C and 55 to 121°C, respectively), high acidity or alkalinity (pH-value below 4 or exceeding 8, respectively), high salinity (1–5 M NaCl), or elevated levels of toxins such as special organic compounds, oxidants, or heavy metals (reviewed by Gomes and Steiner 2004). Only since the last decades, we witness growing attention to such biotechnological processes, which are operated under extreme conditions. A broad product range, encompassing polysaccharides, polyesters, pigments, thermostable "extremozymes", bioactives, compatible solutes including ectoins, and others, are metabolites of commercial interest generated by extremophile organisms (Gomes and Steiner 2004, Torregrosa-Crespo et al. 2017).

The exploitation of both Gram-positive and Gram-negative microbial production strains which could potentially be used as extremophile PHA-biopolyester cellular factories was initiated about thirty years ago, when the high PHA accumulation potential of the extremely osmophilic haloarcheaon *Hfx. mediterranei* was detected (reviewed by Xiang 2016). This extremophile is a metabolically highly versatile organism, and known

for its production of PHA (Xiang 2016), pigments (mainly carotenoids) (Chen et al. 2015), bioactive peptides (halocins) (Cheung et al. 1997), and polysaccharides (Antón et al. 1988, Parolis et al. 1996). Pigments (C50 carotenoids like bacterioruberin) and anionic extracellular polysaccharides (EPS) provide *Hfx. mediterranei* cultures a typical reddish coloration and mucous, slimy character. This product spectrum of *Hfx. mediaterranei* was the ignition spark, which initiated the worldwide quest for other extremophile organisms with high capability for PHA production (reviewed by Fernandez-Castillo 1986).

The subsequent paragraphs provide a compilation of the most significant research activities globally accomplished in the field of PHA production by extremophiles in the past and recently. The review is dedicated to elucidate mechanisms, biological functions, composition, and material features of "extremophile PHA", and provides an outlook for the potential and likeliness of industrial scale PHA production by such organisms. Figure 2 provides a schematic on the most important extremophilic PHA producing species discussed in the chapter.

HALOPHILE PHA PRODUCTION STRAINS

General Features of Halophile Microbes

The expression "halophile" describes the adaptation of living organisms to elevated salt concentrations in their environment. The utilization of halophile microbes as cellular factories for various biotechnological products paves the way for several opportunities: First of all, high salt concentration in cultivation media drastically reduces the risk of contamination by unwanted microbes others than the production strain, which provides a higher stability of fermentation batches. Moreover, thriving halophile cultures in saline media up to high biomass concentrations results in intracellular accumulation of salt, hence, the mitigation of salt from the cultivation medium. This displays a special benefit when cultivating cells in highly saline waste streams, where the salt fraction normally requires disposal. In addition, HCl-catalyzed hydrolysis of various waste materials for generation of carbon-rich substrates for production of PHA and other biomaterials requires neutralization of the hydrolysis cocktail after the acid treatment. The viability of this approach was successfully demonstrated in the past in the case of acid-catalyzed hydrolysis of whey (Koller et al. 2016) and diverse lignocellulosic materials such as bagasse (Cesário et al. 2015, Kulkarni et al. 2015), straw (Ahn et al. 2016, Cesário et al. 2015), spent coffee ground (Obruca et al. 2014), or wood dust (Koller et al. 2015a, Kucera et al. 2017). Neutralization of the acidic mixture after hydrolysis is typically done with NaOH, thus generating considerable amounts of NaCl, which directly acts as part of the required salt fraction in the cultivation medium.

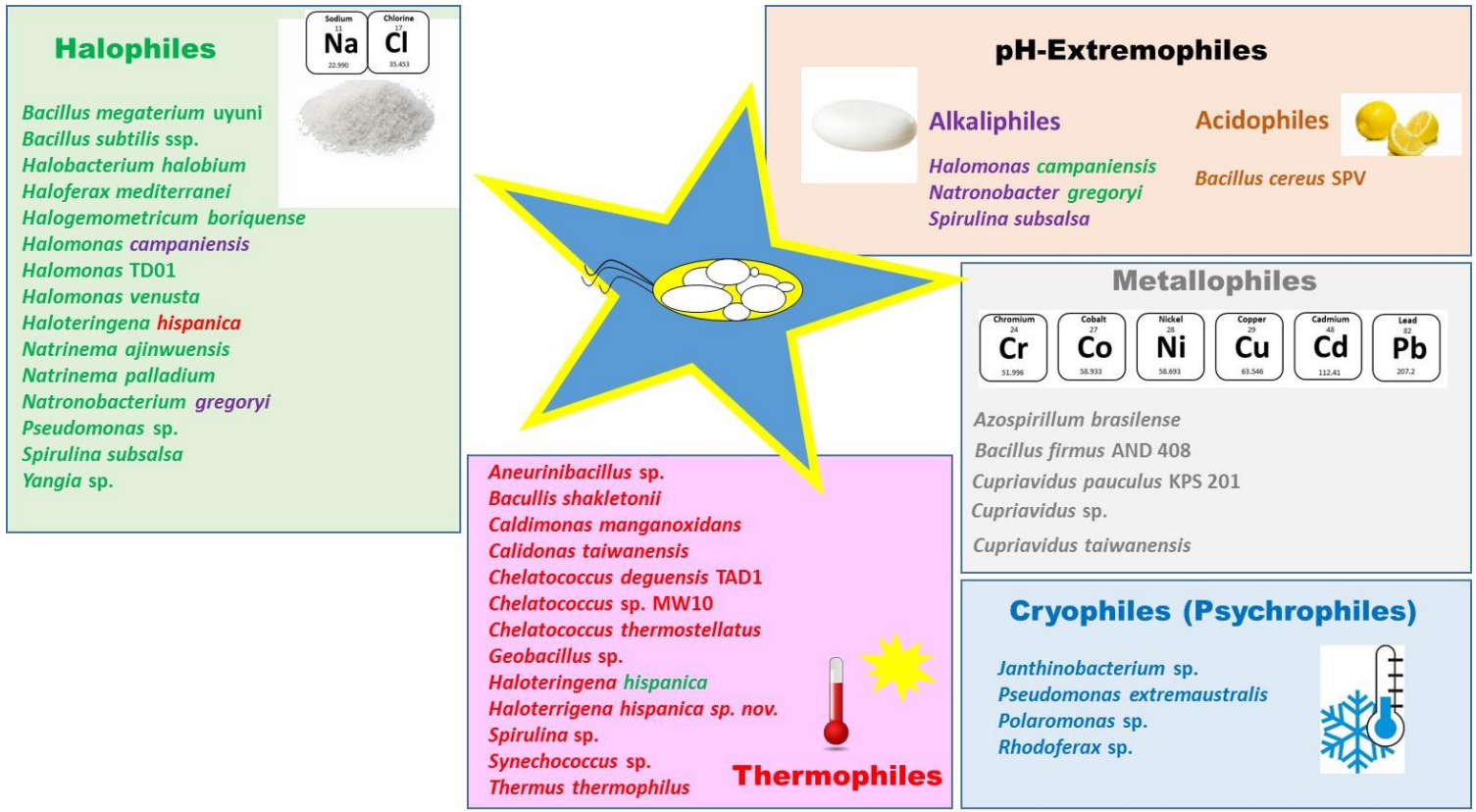

Figure 2. Overview of described extremophile PHA-producers. Note: Tow colours per species name indicate that the strain exhibits two different extremophilicities.

Halophilicity is observed throughout the entire tree of life, and is found among Archaea, Bacteria, and Eukarya. The multifarious strategies of halophiles to cope with high salinity makes clear that adaptation to high salinity is a widespread and well optimized metabolic pattern (reviewed by Rodriguez-Contreras et al. 2016, Torregrosa-Crespo et al. 2017). For example, changing microalgae pigment patterns are a well-known adaptive response to changing environmental conditions such as fluctuating salinity (Borowitzka et al. 1990). Halophiles constitute a phylogenetically highly versatile group of microorganism, which require hypersaline milieus, in which NaCl typically displays the main salt component. According to the salt level that is tolerated by different halophiles, such organisms are traditionally grouped in three categories of halophiles: *halotolerant microbes* tolerate NaCl in a range of 0-15% or up to 2.5 M, respectively, *moderately halophiles* require 1-15% (2.5 M) NaCl for cultivation, whereas and *extreme halophiles* are described to require NaCl concentrations as high as 15-30%, or 2.5 to more than 5 M, respectively. So called "*borderline extreme halophiles*" thrive best in media containing 1.5–4.0 M NaCl, with haloarchaea belonging to the families of *Halobacteriaceae* and *Haloferacaceae* being the most prominent representatives of this group (reviewed by Rodriguez-Contreras et al. 2016, Torregrosa-Crespo et al. 2017).

Different adaptation approaches are used by Nature to enable living organisms to survive and thrive in such extremely saline environments. The most frequently applied strategy resorts to the intracellular accumulation of kosmotropic, compatible organic osmotic solutes; here, no special adaptation of the cell's enzymes to the high salt concentration is needed. Such solutes can be released into the environment when the osmolarity/salinity of the medium decreases. Accumulation of these compatible solutes either occurs via intracellular synthesis, or they are imported into the cell from the extracellular space (Wood et al. 2001). This is in contrast to the alternative adaptation strategy - the so called "salt-in" strategy, where salts like KCl are accumulated inside the cell; this strategy needs adaptation of the enzymatic machinery by making the enzymes salt-resistant, such as generating a surplus of acidic amino acids on the enzymes surface (Oren 2006, 2008). As an example, glucose dehydrogenase of *Hfx. mediterranei* lacks flexible side chains and constitutes a more or less high-ordered multilayered solvation shell (Britton et al. 2006). Also the genome of halophiles often requires special adaptation, as evidenced by the high number of salt-resistant genes found in such organisms (Mirete et al. 2015).

Regarding halophile PHA producers, the complex protective function of PHA carbonosomes against hyperosmotic pressure was recently revealed. Soto et al. (2012) investigated the halotolerant bacterium *Pseudomonas* sp. CT13 and its PHA-negative mutant strain. These authors demonstrated that presence of PHA inclusions results in high intracellular concentration of the PHA monomer – 3-hydroxybutyrate (3HB) - which prevents protein agglomeration under collective salt and temperature stress. In this study, presence of PHA and its monomer as compatible solutes turned out to be essential for the

cells to resist stress provoked by salinity; this was evidenced by increasing PHA productivity in parallel to increasing salt concentrations (Soto et al. 2012). These results were recently confirmed by Obruca et al. (2016) who reported extraordinary chaperoning efficiency of 3HB, which is more than comparable with that of well-established compatible solutes such as hydroxyectoine or trehalose. Thus, water insoluble (and thus not enhancing intracellular osmotic pressure) PHA granules represent a unique reservoir of water soluble compatible solutes which can be used by microbial cells to face osmotic pressure but also other environmental stressors (reviewed by Obruca et al. 2018). Furthermore, presence of PHA granules in bacterial cells also biophysically protect bacterial cells from massive plasmolysis, dramatic damage of cytoplasmic membrane and loss of cellular integrity when exposed to osmotic-up shock (Obruca et al. 2017). Hence, it seems that accumulation of PHA could be an additional strategy adopted by many halophiles to cope with high salinity of the environment.

Gram-Negative Halophile PHA Producers

PHA Production by Haloarchaea

Recent progress in the investigation of biochemical, metabolic, molecular biological, and physiological particularities of haloarchaea reveals that these microbes synthetize a range of commercially significant compounds, such as PHA, as response to the extreme conditions of their habitats; these compounds are produced by both the primary and secondary metabolism of haloarchaea. Generally, haloarchaea are aerobic organisms, although some representatives are reported to use denitrification for terminal electron disposal (reviewed by Torregrosa-Crespo et al. 2017).

The first observation of PHA inclusions in haloarchaea dates back to the early 70ies, when Kirk and Ginzburg (1972) investigated the morphology of *Halobacterium halobium*, a haloarchaeal isolate from the Dead Sea. These morphological studies were carried out by freeze-fracture technique, and revealed the presence of poly(3-hydroxybutyrate) (PHB) granules in this organism. After that, it took some years until halophiles were cultivated to monitor their PHA-accumulation characteristics. Already the first reported PHA production process by an extremely halophile production strain, namely the Archaeon *Hfx. mediterranei*, revealed the potential of such systems to prevent infection by unwanted microbes. *Hfx. mediterranei* was originally isolated at the Iberian coast of the Mediterranean Sea from ponds of a solar saltern near Santa Pola. This strain thrives best in media of salinities exceeding 20% NaCl. Regarding the protection mechanism exerted by *Hfx. mediterrani* to survive high salinity, it is known that the strain accumulates high intracellular KCl concentrations (Rodriguez-Valera and Lillo 1992). This so called “salt-in” strategy is a typical feature of haloarchaea and requires the

adaptation of the strain's proteome to maintain the appropriate conformation and activity of enzymes under conditions approaching salt saturation concentrations (Oren 2008).

One can also profit from the high inner-osmotic pressure of *Hfx. mediterranei* cells to recover PHA granules from cells of this species by a simple strategy: When *Hfx. mediterranei* cells are exposed to aqueous surroundings with salt concentration below 10%, partial cell lysis is observed. If the cells are exposed to salt-free, hypotonic media such as distilled water, the cells even burst due to their high inner-osmotic pressure, and PHA granules are released into the medium (Rodriguez-Valera and Lillo 1992). Based on the different density of the two fractions, the separation of released PHA granules (low density) from the cell debris (high density) can easily be accomplished by decantation or centrifugation; this separation of PHA granules and cell residues can further be accelerated e.g., by using dissolved air floatation. This convenient strategy allows designing extremely simple downstream facilities (Koller et al. 2013b).

From the kinetic point of view, *Hfx. mediterranei* displays faster growth and higher specific PHA productivity if compared to other extremophilic PHA-producers investigated so far. Moreover, PHA produced by *Hfx. mediterranei* is of superior quality regarding its high molecular mass, low polydispersity (P_i), low melting temperature (T_m), and low degrees of crystallinity (X_c) (Lillo and Rodriguez-Valera 1990). In a continuous chemostat cultivation process, which was carried out at a dilution rate *D* of 0.12 1/h, a cultivation temperature of 38°C, and 20 g/L glucose as carbon source, 3.5 g/L PHA was achieved; switching to starch as carbon source, this PHA output was almost doubled to 6.5 g/L (Lillo and Rodriguez-Valera 1990). Most surprisingly, the PHA produced turned out as a poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV) copolyester. This was in huge contrast to the state of knowledge on PHA at that time, since production of PHB-homopolyester was expected from simple carbon sources like starch or glucose. Production of copolyesters containing 3-hydroxyvalerate (3HV) building blocks classically requires the co-feeding of precursor compounds structurally related to 3HV, such as odd-numbered fatty acids (propionic acid, valeric acid, margaric acid, etc.), which contributes considerably to the production costs of PHBHV. At this point, it has to be emphasized that PHBHV copolyesters display enhanced material properties in terms of low melting temperature, lower crystallinity and lower glass transition temperature if compared to the homopolyester PHB, thus facilitating its processibility. Only decades later, PHBHV copolyester production from unrelated carbon sources was also reported for other haloarchaeal strains (as shown below). For *Hfx. mediterranei*, it was revealed that this astonishing metabolic feature is based on particularities of the strain's PHA-biosynthesis pathway; it was shown that the strain accumulates high intracellular pools of the 3HV-precursor compound propionyl-CoA (Han et al. 2013). Additional particularities of *Hfx. mediterranei* PHBHV were revealed in 2006 by Don and colleagues, who showed that this polymer consists of different fractions. Using a chloroform/acetone mixture, these authors separated *Hfx. mediterranei* PHBHV into two fractions, which displayed

different monomeric composition. While the predominant fraction, which amounted to about 93 wt.-% of the total biopolyester, contained about 10.7 mol.-% 3HV and a molecular mass of 569.5 kDa, the 3HV content of the minor fraction was considerably higher (12.3 mol.-%); this fraction had a significantly lower molecular mass of 78.2 kDa, and was soluble even in classical "PHA-non solvents" such as acetone. Both fractions exhibited rather low P_i, and revealed similar values for T_m and glass transition temperature T_g. Using Differential Scanning Calorimetry (DSC), the authors were able to provide an explanation for the occurrence of these two fractions: at heating rates below 20°C/min, two overlapping melting peaks were observed, whereby the relative intensity of these two peaks changed when varying the heating rate, which gave rise to the conclusion that this phenomenon originates from a melt/recrystallization process (Don et al. 2006).

Based on these groundbreaking examinations, further optimization studies on PHA production by *Hfx. mediterannei* were accomplished by researchers all over the world. Many of these investigations were devoted to the utilization of inexpensive carbonaceous raw materials in order to safe substrates, which contribute to the production price and compete with nutritional purposes. Among these "2nd-generation substrates", the application of whey permeate from dairy industry (Koller et al. 2007a,b, Pais et al. 2016), crude glycerol phase (CGP) from biodiesel industry (Hermann-Krauss et al. 2013), extruded rice bran (Huang et al. 2006), enzymatically extruded non-edible starch (Chen et al. 2006), stillage from rice-based ethanol production (Bhattacharyya et al. 2015), olive mill waste water (Alsafadi and Mashaqbeh 2017), vinasse (Bhattacharyya et al. 2012), and others were used for *Hfx. mediterranei* cultivations. Other studies aimed at clarifying the background of *Hfx. mediterranei*-mediated PHA-synthesis on the enzymatic and genetic level (Cai et al. 2012, Feng et al. 2010, Han et al. 2012, Lu et al. 2008), and studied *in vitro* PHA degradation (Liu et al. 2015) by this strain. Moreover, mathematic models were developed to better understand kinetics of PHA production by *Hfx. mediterranei* (Koller et al. 2006), and the kinetics of parallel PHA and by-product synthesis and subsequent re-conversion of the products (Koller et al. 2015b).

Ferre-Guell and Winterburn (2017) investigated the impact of different nitrogen sources (ammonium ion and nitrate) on growth and PHBHV-accumulation kinetics of *Hfx. mediterranei*. Under nitrogen-rich conditions, using a glucose and yeast extract based cultivation medium, 10.7 g/L CDM with 4.6 wt.-% PHBHV were obtained using ammonium as nitrogen source, whereas 5.6 g/L CDM containing 9.3 wt.-% PHBV were generated using nitrate. Surprisingly, the composition of PHBHV was dependent on the nitrogen source applied: in ammonium cultures, the molar 3HV fraction in PHBHV amounted to 16.9 mol-%, which was more than observed for nitrate cultures (12.5 mol-%). Under nitrogen limitations (C/N ratio: 42/1), less biomass was formed, but the mass fraction of PHBHV raised to 6.6 wt.-% (ammonium) or 9.4 wt.-% (nitrate). Moreover, PHBHV from cultures with lower C/N ratios displayed higher 3HV fractions in PHBHV.

In addition, copolyester production only started after a certain cultivation time; 3HV was not detected before a minimum of 0.45 g/L PHA was reached. This means that the mechanisms of copolymer synthesis by *Hfx. mediterranei* are by far not completely elucidated yet; a better understanding of the impact of type and concentration of the nitrogen source will facilitate the design of new PHBHV production process with PHBHV copolyesters of regular structure and pre-defined material properties as final products.

A recent study carried out by Cui and colleagues (2017) examined the temperature effect on growth and PHA biosynthesis by *Hfx. mediterranei*. In their study, the authors developed, validated, and calibrated a mathematical model for the kinetic behavior of this organism at rather low (15, 20, and 25°C) and mesophilic (35°C) temperature. The kinetic coefficients for the model were calculated from experimental results from cultivations in a synthetic medium mimicking a saline (50 g/L NaCl) molasses-based wastewater; these cultivations were carried out in 2.5 L airated and stirred flasks. As major outcome, it was shown that PHA biosynthesis by *Hfx. mediterranei* was strongly temperature-dependent; volumetric PHA productivity ranged from 390 mg/(L h) (15°C) to 620 mg/(L h) (35°C). Maximum specific growth rate ($\mu_{max.}$), maximum specific substrate consumption rate ($qs_{max.}$), and the specific decay rate (k_d) of the strain increased with temperature in accordance to a prediction by an Arrhenius equation plot. $\mu_{max.}$ fluctuated between 0.009 1/h (15°C) and 0.033 1/h (35°C), $qs_{max.}$ between 0.018 g/(g·h) (15°C) and 0.037 g/(g·h) (35°C), and k_d between 0.0048 1/h (15°C) and 0.0089 1/h (35°C). For biomass growth, the estimated activation energy amounted to 58.31 kJ/mol, whereas 25.59 kJ/mol were calculated for substrate consumption, and 22.38 kJ/mol for biomass falloff, respectively. For all investigated temperature conditions, the model turned out to have high predictive character. Further, the composition of the copolyester was independent on the cultivation temperature; the product consistet of 83.3 mol-% 3HB, and 16.7 mol-% 3HV. Temperature of 35°C boosted PHA biosynthesis even under nutritionally balanced conditions, hence without limitation of nitrogen source. Therefore, the authors stated that a temperature regime of about 35°C might be the optimum to run batches for PHA production by *Hfx. mediterranei*.

As mentioned before, *Hfx. mediterranei* excretes an anionic EPS of high molar mass, which provides it's colonies a typical mucous appearance, when grown on solid medium. As revealed by Parolis et al. (1996), this EPS displays an anionic sulfated polysaccharide with a regular trisaccharide repeating unit of one mannose (Man) and two 2-acetamido-2-deoxyglucuronic acid (GlcNAcA) moieties; per trisaccharide unit, one sulfate ester bond is linked to carbon number 3 of the second GlcNAcA moiety. Even low EPS concentrations in aqueous solutions result in drastically increased viscosity; rheological properties of solutions of EPS produced by *Hfx. mediterranei* resemble those of xanthan solutions. Therefore, the haloarchaeal EPS might find application in food technology, e.g., as thickening, gel-forming, or stabilizing agent. Moreover, chemically similar

sulfated EPS produced by algae were already applied as hyaluronidase inhibitor and as anti-allergic, anti-inflammatory, anti-bacterial, antiviral, anti-inflammatory, anti-oxidant, and anti-tumor agents, which makes them interesting for applications in the therapeutic, cosmetic, or nutraceutical field (reviewed by Nazia Auckloo and Wu 2015). However, the high viscosity of EPS-solutions complicates fermentative cultivation of *H. mediterranei* on bioreactor scale by causing difficulties in oxygen input and higher energy demand for the stirring system. Moreover, EPS synthesis in parallel to PHA accumulation causes a loss of carbon source when PHA is the envisaged main product of the bioprocess; hence, it lowers the substrate-to-product yield as a cost-decisive factor.

To investigate the before unknown kinetics for re-use/degradation of both polymeric products (EPS and PHA) by *Hfx. mediterranei* under limitation of exogenous carbon source, the strain was cultivated by Koller et al. (2015b) in a 10 L bioreactor fermentation. A defined minimal medium containing 10 g/L glucose as sole carbon source and a salinity of 150 g/L NaCl was used for the cultivation. After the complete depletion of exogenous carbon source (glucose), the fermentation was stopped. The dense, mucous fermentation broth contained 15.5 g/L biomass (sum microbial protein plus PHA) with 13.0 g/L PHA (46 wt.-%), and 1.31 g/L of excreted EPS. During the entire process, EPS was formed in parallel to PHA, whereby PHA accumulation occurred ten times faster than EPS excretion. After the cultivation was stopped, the fermentation broth was stored under different conditions to study degradation kinetics of EPS and PHA, and the impact of different storage conditions on molar mass of PHA in order to evaluate the requirements for handling product-rich cultivation broth after harvest before product recovery. Again, this study revealed that the cultivation could be run without any sterility precautions. Moreover, only insignificant product losses occurred when the fermentation broth was not subjected towards pasteurization for cell inactivation after the stop of the cultivation process. It was shown that pasteurization is not needed all when the cultivation broth is stored at 4°C after harvest. When pasteurized, the cells can be stored for extended time periods (262.5 h in described experiments) without significant PHA degradation; even storage at RT causes only negligible PHA loss under these conditions. Most importantly, when supplying active *Hfx. mediterranei* fermentation broth with nitrogen source and phosphates, cultivation in shaking flask under aerobic conditions at RT resulted in only slow intracellular degradation of PHA for maintenance and microbial growth, although no other carbon sources apart from EPS and PHA were present. This is in clear contrast to other microbes frequently used for PHA production, e.g., *C. necator*. About 70 wt.-% of the stored PHA remained intact after one day of cultivation. Here, it should be noted that the rather small amount of degraded PHA was allotted to a low molecular mass PHA fraction; degradation of this fraction resulted in an overall increase of the polymer's molecular mass. These outcomes facilitate large-scale cultivation processes with *Hfx. mediterranei*; to preserve produced PHA, fermentation batches do not have to be interrupted immediately after the depletion of exogenous

carbon source. Excreted EPS was not converted by *H. mediterranei* at all even under carbon-limited, aerobic conditions and supplementation with exogenous nitrogen and phosphate source, which indicates that this polymer does not serve as carbon reserve material for *Hfx. mediterranei* (Koller et al. 2015b).

Deeper insights into the impact of salinity on the direction of the carbon flux towards, on the one hand, EPS excretion and, on the other hand, PHA accumulation by *Hfx. mediterranei* were performed by Cui et al. (2017). High NaCl concentrations by trend inhibited EPS excretion, but favored PHA accumulation. As optimal salinity for growth of *Hfx. mediterranei*, the authors suggested the range of 150–200 g/L NaCl based on their experimental data. As NaCl concentration increased from 75 g/L to 250 g/L, EPS productivity by trend dropped from 372 to 320 mg EPS per g cell mass. When the NaCl concentration amounted to 250 g/L, the intracellular PHA mass fraction reached its maximum value of 71.1 wt.-%. These results underlines that a triggered, elevated NaCl concentration considerably stimulates the PHA productivity for *Hfx. mediterranei*, while preventing excessive EPS production; thus, it is possible to influence the intracellular carbon flux to the synthesis of either PHAs or EPS by *Hfx. mediterranei* by monitoring the salinity (Cui et al. 2017).

In context of inexpensive raw materials, *Hfx. mediterranei* is at present regarded the most promising candidate for whey-based PHA production on industrial scale, mainly due to the strain's high robustness and stability of its cultures (Koller et al. 2007a). *Hfx. mediterranei* was shown to thrive well on hydrolyzed whey permeate with high maximum specific growth rates ($\mu_{max.}$) of 0.11 1/h in bioreactor experiments, which displays an excellent value for haloarchaeal organisms. PHBHV was produced on hydrolyzed whey permeate at a maximum specific production rate (q_P) of 0.08 g/(g·h). After optimization of the cultivation conditions, it was possible to increase volumetric and specific productivity to 0.09 g/(L·h) and 0.15 g/(g·h), respectively. Highest biomass concentration and intracellular PHBHV fractions amounted to 16.8 g/L and 73 wt.-%, respectively (Koller et al. 2008a). In subsequent experiments, hydrolyzed whey permeate was used as main carbon substrate, and valeric acid and γ-butyrolactone (GBL) were co-supplemented as precursors for 3HV and 4-hydroxybutyrate (4HB) production, respectively. This substrate supply resulted in the production of a poly(3HB-*co*-21.8%-3HV-*co*-5.1%-4HB) terpolyester of high-quality. After recovering the terpolyester from biomass and detailed characterization of its properties via DSC and molecular mass determination, the authors recommended further investigation of this terpolyester for future medical applications (Koller et al. 2007b).

Because of the high salinity required for efficient *Hfx. mediterranei* cultivations, the risk of microbial infections is definitely insignificant; therefore, sterility precautions typically needed in biotechnological processes can be neglected. No microbial contamination was observed even when cultivation processes were carried out over prolonged periods without using any sterilization precautions at all, hence neither the

bioreactor equipment nor the nutrient medium were sterilized (Hermann-Krauss et al. 2013). An economic assessment of PHA production by *Hfx. mediterranei* on hydrolyzed whey, based on experimental data from a 200 L pilot scale experiment, followed by a solvent-free downstream processing for PHA release from cells, calculated the approximate production price per kg PHA at below € 3 (Koller et al. 2007a).

Apart from using surplus whey as feedstock, *Hfx. mediterranei* is also considered an auspicious candidate strain for production of PHA co- and terpolyesters based on the major side-product of the growing biodiesel production, namely CGP. This was shown by Hermann-Krauss et al. (2013), who achieved a volumetric PHBHV production of 0.12 g/(L h), a PHBHV fraction of 75 wt.-% in biomass, and a 3HV content in PHBHV of 10 mol.-%. Molecular mass determination and thermoanalysis of *Hfx. mediterranei* PHBHV copolyesters based on CGP revealed a weight average molecular mass M_w of 150-253 kDa, a P_i of 2.1 – 2.7, and T_m between 130°C and 140°C for different production batches. Co-feeding the 4HB-precursor GBL together with the main substrate CGP resulted in the biosynthesis of a terpolyester containing 12 mol-% 3HV and 5 mol-% 4HB; this terpolyester revealed lower T_m (two melting endotherms at 122 and 137°C), lower T_g (2.5°C), and higher molecular mass (391 kDa) than the PHBHV copolyesters (Hermann-Krauss et al. 2013). In this context, it should be noticed that haloarchaeal PHA accumulation based on glycerol is no typical feature; many other haloarchaea utilize glycerol for cell growth, but not for PHA biosynthesis (Mahansaria et al. 2017). This was explained by the metabolization of glycerol after phosphorylation in the TCA cycle, thus resulting in microbial growth instead of PHA formation (Lorantfy et al. 2014).

Huang et al. (2006) used extruded rice bran (ERB) and extruded corn starch (ECS) as carbon sources for PHA production by *Hfx. mediterranei*; these experiments were carried out in nutritionally complete media, hence without nitrogen limitation. Because of the strain's deficiency to use rice bran or corn starch directly in their native forms, these substrates were extruded prior to application. Five L bioreactor cultivations were carried out under controlled pH-stat feeding (pH-value maintained constant between 6.9 and 7.1). ERB/ECS mixtures (1/8 g/g) were used as the main carbon source and supplied by a repeated fed-batch feeding regime. As result, 140 g/L cell dry mass (CDM), 77.8 g/L PHA, and 56 wt.-% PHA in CDM were achieved by these experiments. When ECS was used as the sole carbon source, 62.6 g/L CDM, 24.2 g/L PHA, and a mass fraction of 38.7 wt.-% PHA in CDM were obtained. Based on the highly saline conditions used for the cultivation (234 g/L NaCl), the authors assume that, for industrial production of PHA, it might be feasible to operate this repeated fed-batch process for extended periods (Huang et al. 2006).

In a further study, corn starch was subjected towards an enzymatic reactive extrusion process using a single-screw extruder; the enzyme α-amylase was added at quantities of 1–5 g per 100 g wet corn starch mass. The obtained extrudate was applied as carbon source for *Hfx. mediterranei*-mediated PHA production in a pH-stat fed-batch process. In

order to keep the concentrations of carbon- and nitrogen source constant, a mixture of extruded starch and yeast extract was applied in the feed stream in a ratio of 1/1.7 g/g. At the end of the process, the PHA mass fraction in CDM amounted to 50.8 wt.-%. Similar to the other case studies for PHA biosynthesis by *Hfx. mediterranei* discussed above, a PHBHV copolyester was produced. This material contained 10.4 mol-% 3HV in PHBHV, had a T_g of −1.2°C, and, by DSC analysis, revealed two distinguishable melting peaks at 129.1°C and 144.0°C (Chen et al. 2006).

Independent on the applied nutrients, the extreme salinity of the *Hfx. mediterranei* medium, which amounts to 20 – 25 g/L NaCl, calls for the use of distinct bioreactor materials and robust measuring sensors able to sustain such high salt concentrations. Here, high-quality steel, borosilicate glass, or, alternatively, highly stable polymers like poly(ethylene ether ketone) (PEEK) can be used to design bioreactors for cultivation of extremely halophiles (Hezayen et al. 2000). A combination of borosilicate glass and PEEK was used by the company Labfors (CH) to design a lab scale bioreactor for extreme halophiles; this corrosion resistant bioreactor was sued by Lorantfy and colleagues for cultivation of *Hfx. mediterranei* on different carbon sources (glycerol, acetate, and lactate) by different process regimes and a new online technique for biomass measurements based on monitoring the consumption of acid and base during the process. The authors revealed that the substrates are used by the organism in a diauxic manner, and growth of *Hfx. mediterranei* follows the kinetics according to Monod (Lorantfy et al. 2014).

Both for economic and sustainability reasons, it was investigated whether the highly saline side streams of *Hfx. mediterranei* cultivation processes can be recycled by using them in follow-up fermentation batches. In fact, the disposal of salt after completion of the fermentation creates a real environmental threat, because current ecological standards do not allow discharging total dissolved solids (TDS) exceeding 2,000 mg/L in wastewater. Therefore, recycling experiments were carried out, demonstrating the viability of using spent fermentation broth of PHA-production by *Hfx. mediterranei* on hydrolyzed whey for subsequent fermentation batches; a considerable share of fresh saline cultivation medium can be substituted by spent fermentation broth. Moreover, salty PHA-free cell debris, which remains after PHA recovery by hypotonic treatment from *Hfx. mediterranei* biomass form previous cultivations, can be used to replace about 29% of yeast extract, which is normally used as expensive nitrogen- and phosphate-rich growth additive for efficient cultivation of *Hfx. mediterranei* (Koller 2015). Bhattacharyya and colleagues, who used waste stillage from the rice-based ethanol production for *Hfx. mediterranei*-mediated PHB production, followed a similar strategy (Bhattacharyya et al. 2014). In shaking flask cultivations, these authors reached 16.4 g/L PHA, a PHA mass fraction in CDM of about 70 wt.-%, a volumetric PHA productivity of 0.17 g/(L h), and a conversion yield of substrate to PHA of 0.35 g/g. Similar to the above experiments, a PHBHV copolyester with a 3HV fraction of 15.3 mol-% was synthetized

by *Hfx. mediterranei*. As the environmentally most advantageous outcome of their study, the authors report a notable reduction of both the chemical and biochemical oxygen demand (COD and BOD) of stillage, which amounted to about 85%. The TDS content in the finally dischargeable water amounted to 670 mg/L, which is only about a third part of the allowed concentration. For PHA release from biomass, also this study resorts to simple cell lysis in hypotonic media; the crude product, consisting of PHA granules covered by a membrane composed of proteins, lipids, and (lipo)polysachcarides, was further refined by purification with organic solvents and sodium dodecyl sulfate (SDS) (Bhattacharyya et al. 2014).

Only recently, Han and colleagues reported the production of blocky structured copolyesters (*b*-PHA) by *Hfx. mediterranei*. Such materials consist of alternating PHB and poly(3-hydroxyvalerate) (PHV) homopolyester blocks, which are linked to randomly distributed PHBHV copolyester blocks. These authors demonstrated how this *b*-PHA production can be optimized by glucose and valerate co-feeding. Due to its "blocky" nature and its high 3HV fraction, *b*-PHA produced by *Hfx. mediterranei* displays exceptional material properties, e.g., lower crystallinity or enhanced Young's modulus. Moreover, this *b*-PHA was also subjected towards biomedical investigation. Here, it turned out that the *b*-PHA displayed superior platelet adhesion and accelerated blood clotting if compared to randomly distributed PHBHV; consequently, *b*-PHA from *Hfx. mediterranei* might be an auspicious candidate for medical application (Han et al. 2015).

During the recent years, additional haloarchaea apart from *Hfx. mediterranei* were isolated from diverse environments. Salgaonkar et al. (2013) screened seven extremely halophilic isolates, all belonging to the Archaea domain, from Indian brine and sediment samples of solar salterns. These isolates were able to grow and to produce PHA in a synthetic saline medium with a NaCl concentration of 20 wt.-%. Phenotypic and genotypic characterization revealed that six of these isolates, labeled as TN4, TN5, TN6, TN7, TN10 and BBK2, belong to the genus *Haloferax*. On the contrary, isolate TN9 was classified as the strain *Halogeometricum borinquense*. *Hgm. borinquense* (TN9) was subjected towards kinetic investigation to study its growth and PHA accumulation characteristics; it turned out that this organism shows highest PHA productivity already during the exponential growth phase, hence, it can be classified as a so-called "growth-associated PHA-producer", which does not require limitation of growth-essential medium components to boost PHA production, as known from most industrially implemented PHA producers. After 5 days of cultivation, a mass fraction of about 14 wt.-% of the homopolyester PHB in CDM was reported (Salgaonkar et al. 2013). Soon later, the same authors isolated another haloarchaeon from solar salterns of Marakkanam in Tamil Nadu, India. This strain was called *Halogeometricum borinquense* E3, and displayed PHBHV copolyester biosynthesis on a highly saline medium containing glucose as sole carbon source in four days shaking flask cultivation setups, as shown by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and proton nuclear magnetic

resonance spectroscopy (^{1}H-NMR) characterization. The final PHBHV fraction in CDM amounted to 74 wt.-%, with about 21.5 mol-% 3HV being detected in the copolyester (Salgaonkar et al. 2015).

As a follow up of these studies, the same group of researchers used several haloarchael species for PHA production on hydrolyzed sugarcane bagasse (SCBH) as an abundant lignocellulosic agro-industrial surplus product (Salgaonkar and Bragança 2017). Among the different wild-type haloarchaea screened by Nile Red, the strain *Hgm. borinquense* E3, the organism described above, displayed superior growth and PHA accumulation in comparison to the other organisms investigated in parallel, namely *Haloferax volcani*i BBK2, *Haloarcula japonica* BS2, and *Halococcus salifodinae* BK6. Using 25% or 50% of hydrolyzed SCB, respectively, in a highly saline medium (200 g/L NaCl), *Hgm. borinquense* E3 revealed PHA fractions in CDM of 50.4% (25% SCB) and 45.7%, respectively (50% SBC), and specific productivity (q_p) of 3.0 mg/(g·h) (25% SCB) and 2.7 mg/(g·h) (50% SBC); the cultivations were carried out on shaking flaks scale at 37°C. After about six days of cultivation of the setups with 25% SCBH, PHA was chloroform-extracted from oven-dried haloarchaeal biomass; characterization of PHA was carried out by a crotonic acid assay according to Slepecky and Law (1960), XRD, DSC, FTIR, and ^{1}H-NMR. It was observed that the biopolyester obtained from SCBH hydrolysate was a PHBHV copolyester with 13.29 mol-% 3HV monomers in PHA (Salgaonkar and Bragança 2017).

Similar experiments were carried out by Danis and colleagues (2015), who investigated five extremely halophilic archaeal isolates, aiming to explore among them the strain with highest capacity for PHA production. In these experiments, a range of inexpensive carbonaceous feedstocks like, e.g., corn starch, sucrose, waste of apples, melons and tomatoes, or whey, were individually tested for their impact on PHA production of each microbial isolate. Corn starch was identified as the most promising substrate for PHA productions among these feedstocks. Comparing the five investigated halophile strains, the isolate 1KYS1 showed the best performance in PHA biosynthesis. Further, the narrow relationship of 1KYS1 to the genus *Natrinema*, and, within this genus, a very close relation to the species *Natrinema pallidum* JCM 8980 was revealed by comparative 16S rRNA gene sequence analysis. Using starch as the only carbon source, a PHA fraction in CDM of 53.14 wt.-% was reached by 1KYS1. The produced PHA was identified as the copolyester PHBHV, which was accumulated as large and uniform PHA granular inclusions, as it was observed via transmission electron microscopy (TEM). The PHBHV copolyester isolated from 1KYS1 biomass was blended with low molar mass poly(ethylene glycol) in order to prepare biocompatible films to be used in drug release studies; for these studies, Rifampicin was used as model bioactive substance It was demonstrated that conditions resembling physiological conditions in the human body (37°C and a pH-value of 7.4) resulted in best Rifampicin delivery efficiency (Denis et al. 2015).

The extremely halophile Archaeon *Natrinema ajinwuensis* RM-G10 (synonym *Natrinema altunense* strain RM-G10), isolated by Mahansaria and colleagues in 2015 from Indian salt production pans, turned out to store 61.02 ± 0.68% PHA in its CDM in 72 h repeated batch cultivations; volumetric PHA productivity after process optimization amounted to 0.210 ± 0.001 g/(L h); glucose was identified as the carbon source of choice for PHA production by this strain, whereas cultivation on glycerol resulted in cell growth, but the culture did not accumulate PHA. The produced PHA was characterized by electron microscopy, GC-FID, GC-MS, thermogravimetry, DSC, XRD, FTIR, and NMR. These tests showed that the produced biopolyester was, similar to PHA produced by other haloarchaea summarized in this chapter, a PHBHV copolyester with a 3HV-fraction of 13.93 mol-%. The copolyester revealed a X_c of 35.45%, a T_g of −12.3°C, two melting endotherms with peaks at T_{m1} = 143°C and T_{m2} = 157.5°C, and a decomposition onset temperature of (T_d) of 284°C (Mahansaria et al. 2017).

Another haloarchaeon, *Halogranum amylolyticum* TNN58, was described by Zhao and colleagues (2015) as a powerful producer of PHBHV copolyester. Similar to other haloarchaea, PHBHV production by this strain again occurred by simply using unrelated carbon sources (glucose) without the need to supply 3HV-related precursors. Using transmission electron microscopy (TEM), the authors spotted the pronounced accumulation of PHA carbonosomes in the haloarchaeal cells. Via GC-MS and ^{1}H NMR, the PHA was unambiguously identified as PHBHV copolyester. Most importantly, the 3HV fraction in PHBHV surmounted 20 mol.-%, which up to now displays the highest 3HV fraction in PHBHV produced from structurally unrelated substrates by wild-type strains. Medium optimization revealed that nitrogen-limited medium performed superior for PHBHV production by this strain than diverse nutrient-rich media. Moreover, glucose turned out as the best among the tested carbon sources such as glycerol, benzoic acid, lauric acid, butyric acid, starch, casamino acids, and acetate. In addition, PHBHV accumulation occurred in a growth-associated way. PHBHV production by *H. amylolyticum* was carried out under controlled conditions in 7.5 L bioreactors in feedbatch mode. Four substrate pulses of glucose solution were added after 64, 90, 114, and 144 h. When the cultivation was finished after about 188 h, a concentration of CDM and PHBHV of 29 g/L and 14 g/L, respectively, was reached, which corresponds to a volumetric productivity of about 0.074 g/(L·h) for the entire process (Zhao et al. 2015).

Twenty haloarchaeal isolates were investigated by Legat et al. (2010) by staining with Sudan Black B, Nile Blue A, and Nile Red to screen for the presence of PHA inclusions. TEM and ^{1}H-NMR spectroscopy were applied to visualize PHA granules and the monomeric composition of PHA in the haloarchaeal biomass. Among the halophile isolates, PHA production was evidenced for *Halococcus morrhuae* DSM 1307^T, *Halococcus saccharolyticus* DSM 5350^T, *Halococcus salifodinae* DSM 8989^T, *Halococcus dombrowskii* DSM 14522^T, *Halococcus hamelinensis* JCM 12892^T, *Halococcus qingdaonensis* JCM 13587^T, *Halobacterium noricense* DSM 9758^T,

Halorubrum coriense DSM 10284^T, *Halorubrum chaoviator* DSM 19316^T, and *Halorubrum chaoviator*. The strains were cultivated in complex media from literature and mineral media containing 200 g/L NaCl. This study constitutes the first report on PHA production by representatives of the genus *Halococcus*. All of these PHA producers except *Hcc. saccharolyticus* produced the copolyester PHBHV; in the case of PHA from *Hcc. saccharolyticus*, only 3HB monomers were detected (Legat et al. 2010).

The elucidation of genes responsible for haloarchael PHA biosynthesis is another emerging field to better understand and optimize PHA production by extremophiles. Haloarchaeal genes encoding the homologues of Class III PhaC synthase, the enzyme catalyzing the polymerization of hydroxyacyl-CoA to PHA in strains like *Allochromatium vinosum*, were first found in the genomes of *Haloarcula marismortui* ATCC 43049 and *Haloquadratum walsbyi* DSM 16790 by Baliga et al. (2004) and Bolhuis et al. (2006), respectively. The expression profile of genes encoding the enzymes, which catalyze haloarchaeal PHA synthesis, were for the first time investigated experimentally by Han and associates (2007). By studying the haloarchaeon *Haloarcula marismortui*, an extremophile organism originating from the Dead Sea, these authors found that this strain was able to store PHB in CDM up to a mass fraction of 21 wt.-%; the cultivation experiments were carried out in minimal medium containing excessive amounts of glucose as carbon source. Most importantly, the authors identified *phaEHm* and *phaCHm*, two neighboring genes encoding two subunits of a Class-III PHA synthase by molecular characterization of the $phaEC_{Hm}$ operon. These genes are directed by only one single promoter, which is located 26 bp upstream of the transcriptional start position. It was demonstrated that expression of the two genes occurs in a constitutive manner, both nutritionally balanced conditions during microbial growth, and under conditions of nutrient limitation. It is remarkable that *PhaCHm* is strongly connected to the PHA granules, which is in contrast to the non-bound $PhaE_{Hm}$ gene. Transferring *phaEHm* or *phaCHm* genes into the strain *Haloarcula hispanica*, an organism widely used in haloarchaeal studies, e.g, for isolating haloviruses, which harbors $phaEC_{Hh}$ genes of high homology, considerably increased PHB synthesis in the new recombinant *Har. hispanica*. Especially the co-expression of both genes leads to highest PHB biosynthesis; *vice versa*, knocking out the $phaEC_{Hh}$ genes in *Har. hispanica* completely terminates PHA formation. By transferring $phaEC_{Hm}$ genes into these knockout mutants, it was possible to completely restore PHA synthase activity and the PHA accumulation capability of the organism. These studies demonstrated for the first time the significance of *phaEC* genes for archaeal PHA biosynthesis. As a follow up, Ding and colleagues sequenced the genome of a *Har. hispanica* ssp.; surprisingly, these authors detected substantial differences from the previously reported gene sequence of the model organism *Har. hispanica* ATCC 33960 (Ding et al. 2014), the strain used in the before described molecular characterization studies of Han et al. (Han et al. 2007).

Gram-Negative Halophile Eubacteria as PHA Producers

Not only halorachaea are reported as halophile PHA producers, also numerous eubacteria thrive well under high salinity, and accumulate reasonable quantities of PHA under such conditions. Singh (2014) reported the isolation of various halotolerant and halophilic strains of agricultural origin. The isolates were cultivated on different solid agar-agar media with a salinity of 2 M NaCl. Among the tested media, solid Luria Bertani (LB) medium revealed best growth of the organisms in comparison to Nutrient agar or trypticase agar; in addition, significant growth was also observed using the selective medium DSC97. After optimization of the cultivation conditions, the strains were characterized biochemically (citrate utilization, catalase test, etc.) and by different staining techniques using methylene blue, Gram staining, Sudan Black, etc. The isolated organisms, which revealed highest PHA-production potential, were identified as a Gram-positive *Bacillus subtilis* ssp. and a Gram-negative *Pseudomonas* sp. by molecular characterization via 16S rRNA analysis (Singh 2014).

As a particular group of Gram-negative bacteria, cyanobacteria are characterized by their photosynthetic activity and their diazotropy, hence, fixation of molecular nitrogen. Cyanobacteria can be found in almost all terrestrial or aquatic environments, and, besides being versatile cell factories for a range of pigments, bioactives, extracellular polysaccharides, etc., are to an increasing extend investigated as producers of "third generation PHA" (Drosg et al. 2015, Koller and Marsalek 2015, Troschl et al. 2017). In this context, Shrivastav et al. (2010) examined the cyanobacterium *Spirulina subsalsa*, a marine photoautotroph organism originally isolated from samples collected at the Indian coast. This cyanobacterium showed increased PHA production when exposed to elevated salinity levels of 50 g/L NaCl, indicating the protective function of PHA granules against salinity stress. The accumulated biopolymer, a PHB homopolyester, was characterized by DSC, FTIR, NMR, and thermogravimetric analysis (TGA).

Apart from cyanobacteria, also other Gram-negative species are considered being interesting PHA producers. Yue et al. (2014) were the first who cultivated halophile eubacteria on larger scale to investigate PHA production and to profit from restricted sterility requirements when farming such organisms. These authors isolated the halophile and at the same time alkaliphile eubacterial strain *Halomonas campaniensis* LS21 from sludge and plant residues samples, which were collected from the Chinese Dabancheng salt lake. This strain was cultivated in a non-sterile, open, continuously operated process dedicated to PHA production. This process was based on alkaline seawater to preserve fresh water, and on carbon-rich artificial kitchen waste to avoid the use of substrates of nutritional relevance. The artificial kitchen waste mainly consisted of carbohydrates, proteins, and lipids. PHA was chosen as the strain's model bioproduct when studying the possibility of long-term cultivation of industrially relevant production strains in open, non-sterile systems. For further process optimization, the strain's accessibility towards genetic engineering was studied. For this purpose, *H. campaniensis* LS21 was equipped

with additional *phbCAB* genes encoding the PHB biosynthesis enzymes. Both the wild type strain and the recombinant construct were cultivated for 65 days in an open, continuously operated process in the artificial seawater- and kitchen waste-based nutrient medium. Conditions of elevated salinity and extreme alkalinity (27 g/L NaCl and pH-value 10, respectively) and a mesophile temperature of 37°C were identified as the strain's optima for cultivation. Under these conditions, the genetically engineered *H. campaniensis* LS21 reached a PHB mass fraction in CDM of about 70 wt.-%, whereas PHB accumulation by the wild type strain did not exceed 26 wt.-% in CDM. As a major outcome, no microbial contamination was observed during the cultivation of both the wilde-type and the genetically engineered strain; hence, the cultures remained monoseptic despite the open, non-sterile process regime. The utilization of the complex substrates in the artificial kitchen waste by the strain was enabled by the excretion of extracellular hydrolytic enzymes. Also during prolonged cultivation, the recombinant cells maintained the plasmid that carried the *phbCAB* genes for PHA biosynthesis, signifying the viability of using this recombinant strain for long-term continuous processes. Combined with its advantageous amenability for genetic manipulation, the strain *H. campaniensis* LS21 indeed constitutes a potent candidate for cost-, water-, and energy-efficient production of PHA, extremozymes and other valuable products from inexpensive raw materials (Yue et al. 2014).

In the context of alkaliphily, as described for *H. campestris*, also cyanobacterial strains were found which preferably accumulate PHA under elevated pH-values. This is especially the case for *Spirulina platensis*; for this organism, the optimum pH-value for both PHA production and degradation is reported in the rather strong alkaline range of 9 to 11 (Jau et al. 2005). An additional haloalkaliphile, *Natronobacterium gregoryi* NCMB 2189^T, also a representative if the Archaea domain, was identified as PHA producer by Legat et al. This organism was cultivated at a pH-value of 9.0 and 200 g/L NaCl, and revealed accumulation of the copolyester PHBHV (Legat et al. 2010).

Another halophile organism, the proteobacterium *Halomonas* TD01, was isolated from the Chinese Aydingkol salt lake and cultivated by Tan and colleagues (2011) in an open, non-sterile, continuous process similar to the experiments carried out by Yue et al. (2014) with *H. campaniensis* LS21. Glucose was used as the sole carbon source for 56 h fed-batch cultivations at 37°C, 50-60 g/L NaCl, and a pH-value of 9.0; here, the strain reached CDM concentrations and intracellular PHA fractions in CDM of 80 g/L and 80 wt.-%, respectively. An open, non-sterile, continuous two-stage process, consisting of two parallel stirred tank bioreactors with initial working volumes of 3 L and 1L, respectively, was operated for two weeks; in the first stage, based on a glucose- and nitrogen-rich saline medium, the culture reached an average CDM of 40 g/L and a PHA mass fraction in CDM of about 60 wt.-%. From this first stage, the cultivation broth was continuously pumped into a second stage, were nitrogen was limited to boost PHA production. Despite the fact that this transfer results in dilution of the CDM

concentration, a constant PHA mass fraction between 65 and 70 wt.-% in CDM was reached in the second stage. In the first stage, the conversion yield of glucose to PHA amounted to 0.20 - 0.30 g/g; surprisingly, in the second stage, this value exceeded 0.50 g/g. The cultivation broth was removed from the second bioreactor in a way to keep a constant volume of 3 L volume in this stage. After biomass harvest by centrifugation, the supernatant (spent fermentation broth) was collected, treated at 50–60°C and pH 10, cooled and recycled to the feed streams for stage 1 and 2 (Tan et al. 2011). This recycling of spent fermentation broth can be considered a continuous process of reusing saline waste stream in comparison to the batch-recycling of spent fermentation broth as demonstrated in the case of *Hfx. mediterranei* (Koller 2015).

The same organism, *Halomonas* TD01, was later genetically engineered by the same group of researchers. The gene encoding 2-methylcitrate synthase, the enzyme responsible for depletion of propionyl-CoA, was knocked out, which resulted in almost doubling of the propionate-to-3HV conversion yield, causing a two-fold increase of the 3HV molar fraction in randomly distributed PHBHV copolyesters. In a defined medium containing glucose as main carbon source and 0.5 g/L propionate as 3HV-precursor, 70 wt.-% of PHBHV were reached in CDM; in this case, the molar 3HV fraction in PHBHV amounted to 12%. Further engineering of the strain involved the deletion of the genes encoding for three PHA depolymerases in order to avoid intracellular PHA degradation, which typically occurs in later phases especially of large-scale cultivation setups; this deletion did not result in a considerable increase of the overall PHA production. Cultivation studies were performed on 500 L pilot scale; in these large-scale setups, the genetically engineered *Halomonas* TD01 reached a CDM of 112 g/L and a PHB mass fraction in CDM of 70 wt.-%, when glucose was applied as the sole carbon source. By co-feeding propionate, 80 g/L CDM with a PHBHV mass fraction of 70 wt.-% were obtained; here, the copolyester contained 8 mol-% 3HV. For shaking flask scale experiments, mass fractions of PHB in CDM of even 92 wt.-% were reported, together with significantly improved glucose-to-PHA conversion yields. Further engineering experiments were performed with the aim to increase the genetic stability of the engineered *Halomonas* TD01 strain by partial inhibition of its DNA restriction/methylation system and, in order to induce the expression of genes responsible for multiple pathways, the creation of the stable conjugative plasmid called *pSEVA341*. The new construct *Halomonas* TD08, characterized by the insertion of 2-methylcitrate synthase and the knockout of three depolymerases, accumulated up to 82 wt.-% PHBHV in biomass. For follow-up studies, the authors suggested the testing of stability and performance of these auspicious genetically engineered halophile microbial strains in long-term continuous cultivation processes (Tan et al. 2014).

Only recently, Stanley et al. (2017) reported PHA biosynthesis by the moderate halophile proteobacterium *Halomonas venusta* KT832796, an organism isolated from Indian marine environments. The authors optimized the nutrient supply and cultivation

parameters for farming the strain under controlled conditions in 2 L laboratory bioreactors. Regarding the moderate halophilic nature of the strain, the authors carried out studies to evaluate the optimum salt concentration for PHA production with this organism; investigating a NaCl range between 1.5 and 15% NaCl, the lower concentration level (1.5%) turned out as optimum value for the cultivation setups. Preliminary studies were carried out the evaluate the impact of different nitrogen sources (ammonium chloride, ammonium sulfate, ammonium nitrate, ammonium phosphate, ammonium acetate, ammonium citrate, and ammonium oxalate) and carbon sources (fructose, glucose, glycerol, lactose, and sucrose); ammonium citrate and sulfate were identified as the best nitrogen sources, whereas glucose performed as the most suitable carbon source. Starting with studies on shaking flask scale, the strain was able to produce 3.52 g/L CDM containing 70.56 wt.-% PHA when applying a glucose (carbon source) to ammonium citrate (nitrogen source) ratio of 20:1. When applying a fed-batch cultivation regime, it was beneficial to switch to ammonium sulfate for nitrogen supply. On bioreactor scale, the authors investigated different feeding strategies such as fed-batch feeding as response to changing pH-values, and various ways of pulse feeding in order to increase PHA productivity. The pH-linked feeding approach improved PHA concentration to 26 g/L; however, a major part of the carbon flux was redirected towards formation of non-PHA biomass, which resulted in rather modest PHA fractions in CDM of about 39%. A feeding mode using a high concentration single pulse resulted in a maximum PHA concentration of 33.4 g/L, which is related to a PHA fraction in CDM of 88.12 wt.-%. If compared to batch setups, this corresponds to an almost nine-fold increase of PHA production (Stanley et al. 2017).

Coastal marine sites and estuarine systems often display high salinity and therefore are a rich pool to trace halophile microorganisms. Lau and co-workers (2017) deciphered the genome sequence of a halophile eubacterial isolate from Malaysian Matang Mangrove soil sediments. This isolate was identified as *Yangia* sp. CCB-MM3. The work accomplished by the authors resulted in the first entirely deciphered gene sequence of a *Yangia* sp., which belong to the family of Rhodobacteraceae. The Malaysian Mangrove bionetwork and ecosystem is characterized by both marine conditions and by rivers. The genome of *Yangia* sp. CCB-MM3 encompasses two chromosomes and five plasmids; this genome has an entire size of 5.522,061 bp, and an average GC content of 65%. The presence of a propionyl-CoA synthesis pathway and a PHA biosynthesis gene cluster in *Yangia* sp. CCB-MM3 was confirmed by genome sequence analysis. *Yangia* sp. CCB-MM3's ability to accumulate PHA was experimentally studied and also confirmed *in vitro*. The authors of the study assumed that the strain's genome sequence of this strain will substantially subsidize the understanding of the physiological and metabolilic particularities of the genus *Yangia* in general, and should be compared with the genome of described and new representatives of the family of Rhodobacteraceae (Lau et al. 2017).

An interesting advantage of employing halophilic bacteria for PHA productions is associated with their strategy to cope with high external salinity. To balance osmotic pressure, numerous Gram-negative halophilic prokaryotes accumulate substantial amount of small organic molecules called compatible solutes such as trehalose, betaine, amino acids or ectoines. Intracellular concentration of these compounds can reach up to molar values, and bacterial cells spontaneously excrete compatible solutes when exposed to hypotonic conditions which simplify their isolation and purification. Due to their general protective functions and chaperoning activity, compatible solutes find numerous applications in cosmetics, medicine, food industry or biotechnology (Sauer and Galinski 1998). Mothes et al. reported efficient co-production of ectoines and PHA employing *Halomonas elongata.* In the presence of 100 g/L NaCl, the *H. elongata* culture accumulated 50 wt. % of PHA and up to 14% of ectoines which confirmed viability of the concept of PHA and ectoines coproduction (Mothes et al. 2008). Similarly, Guzmán et al. studied the co-production of ectoines and PHA using bacterium *Halomonas boliviensis*. The authors developed a process consisting of two fed-batch cultivations. The first cultivation was aimed at reaching high cell density in a medium with 45 g/L of NaCl and without nitrogen or phosphate limitation. In the second cultivation, NaCl concentration was increased to 75 g/L to trigger ectoine synthesis, while nitrogen- and phosphate sources were fed only during the first 3 h and then stopped to favor PHB accumulation. The process provided a PHB content in bacterial biomass of 68.5 wt.-% with productivity about 1 g/(L·h) and ectoine concentration and content of 4.3 g/L and 7.2 wt.-%, respectively (Guzman et al. 2009).

Gram-Positive Halophile PHA Producers

Reports on PHA production by Gram-positive microbes are generally scarce if compared to the myriad of reports on PHA production by Gram-negatives. This is in conflict with the fact that PHA biosynthesis was originally detected for a Gram positive strain, namely *Bacillus megaterium*, by Lemoigne already in the 1920ies (Lemoigne 1926). Afterwards, PHA production by *Bacilli* became more or less neglected by the scientific community. This situation changed only about ten years ago, when a renaissance of members of the genus *Bacillus* as PHA producers started by detecting new powerful PHA producers in this genus, such as *Bacillus* sp. JMa5, which displays an excellent PHA producer on molasses (Wu et al. 2001), *Bacillus cereus*, a strain that accumulates PHA in quantities up to half of its CDM (Halami 2008), or *Bacillus* sp. IPCB-403, where PHA accumulation reaches up to 70% of CDM (Dave et al. 1996). Large-scale production of PHA was reported by the new Gram-positive strain *Bacillus cereus* SPV (Valappil et al. 2007, 2008). Gram-positive bacteria do not produce lipopolysaccharides (LPS), a group of inflammatory active endotoxins produced in the

cell wall of Gram-negative bacteria. When PHA is isolated from Gram-negative biomass, LPS are typically co-isolated together with the biopolyester, hence, LPS production severely hampers the *in vivo* application of PHA from Gram-negative organisms, e.g., its use for production of implants, surgical sutures, etc. (Luef et al. 2015, Zinn et al. 2001).

The currently rather small number of Gram-positive microbes which are reported to accumulate substantial quantities of PHA in saline milieus is represented in this review by the isolate *Bacillus megaterium* uyuni S29. This organism was originally isolated in Bolivia from water and mud samples of the Uyuni salt lake, and first described as PHA producer in 2013 by Rodríguez–Contreras and colleagues. Figure 3 shows a light microscope picture of this organism, with PHA granules ("carbonosomes") being well visible as light-refractive inclusion bodies. Shaking flask scale cultivation on glucose as sole carbon source resulted in production of PHB homopolyester; surprisingly, this PHB constituted a blend of different polyester fractions of different molecular masses (Rodríguez–Contreras et al. 2013a). Later, this strain was reported to display high PHB production capacity in minimal nutrient media typically used for biotechnological purposes. On 3 L bioreactor scale, 8.5 g/L PHB were produced, corresponding to a volumetric productivity of 0.25 g/(L·h) and an intracellular PHB fraction of 30 wt.-%; these results constitute the up to now best outcomes for PHA production by a member of the genus *Bacillus* (Rodríguez–Contreras et al. 2013b). Later, the impact of fluctuating salinity conditions on growth kinetics and PHA formation of isolate *Bacillus megaterium* uyuni S29 was studied using cultivation media with different NaCl concentrations. These concentrations were chosen in accordance to the salinities prevailing in the natural salt lake milieu of the organism (Rodríguez–Contreras et al. 2016). The strain exhibited surprising flexibility in terms of responding to changing salinity; best outcomes for both growth and PHA biosynthesis were obtained at NaCl concentrations of 45 g/L. *Bacillus megaterium* uyuni S29 even thrives well at extremely high salinities of 100 g/L NaCl, and PHB accumulation was observed at NaCl concentrations up to 250 g/L. As another benefit of this organism, no spore formation was observed under conditions favoring PHA production. Sporulation typically hampers the utilization of Gram-positive strains for PHA production, because it causes the partial loss of the carbon source, hence, lower substrate-to-PHA conversion yields. In the case of *Bacillus cereus* SPV, the strain isolated and described by Valappil et al., sporulation was suppressed by acidifying the pH-value of the medium to pH-values between 4.5 and 5.8; this acidification also increased the yield of PHA biosynthesis (Valappil et al. 2008). Acidification was not necessary in the case of the halophile *B. megaterium* uyuni S29. Overall, *B. megaterium* uyuni S29 looks attractive not only as production strain to provide PHA for biomedical applications, but also for bioremediation such as the treatment of highly saline wastewater (Rodríguez–Contreras et al. 2016).

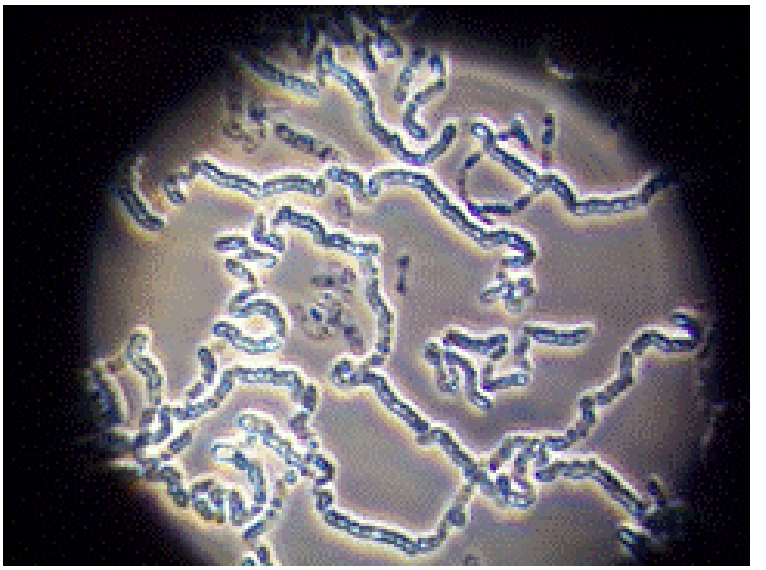

Figure 3. *Bacillus megaterium* uyuni S29 observed in the light microscope. PHA granules are visible as light-refractive inclusions (Picture generated and provided by courtesy by A. Rodriguez-Contreras).

Tracing Halophilic PHA Producers

A variety of different techniques are reported for detecting PHA inclusions in new microbial isolates (Koller and Rodríguez–Contreras 2015). Regarding halophile strains, previously available methods to trace PHA do not perform as precisely as required, they often provide false positive results, e.g., when using Nile Red staining because of the accumulation of lipophilic inclusions, or considerable intracellular PHA accumulation is needed prior to the screening process. To solve this problem, a new method was developed by Mahansaria et al. (2015). This method was based on the amplification of the highly conserved *phaC* gene region with about 280–300 bp, which encodes the Class-III PHA synthase of halophile organisms. The primers *codehopCF* and *codehopCR* were used for the amplification; these primers were originally developed in 2010 by Han et al. A total of nine already known haloarchaea and halobacteria, and 28 new halophile strains isolated at the Indian coast, where used to test this new method. Among these strains, 28 turned out to be *phaC*-positive, and eight strain were *phaC*-negative, although previous Nile Red staining gave false positive results. By 16S rRNA analysis, nine new haloarchaeal strains and nine new halobacteria were identified as potential PHA producers. Further, multiple sequence alignment of the *phaC* gene-derived amino acid sequences was carried out; this technique revealed that only seven amino acid residues were conserved within all four *phaC*-encoded PHA synthase classes, but 61 amino acids were matching amongst the synthase encoded by *phaC* specific to the examined halophile strains. All organisms, which gave *phaC*-positive results in the amplification test, were

also able to accumulate PHA in nutritionally limited medium, whereas none of those strains, which gave negative results when screening for *phaC* showed PHA production in the cultivation experiments. According to the authors, this new method can be considered as highly precise due to the elimination of false positive results as obtained by Nile Red staining (Mahansaria et al. 2015).

CRYOPHILIC (PSYCHROPHILIC) PHA PRODUCERS

General Features of Cryophilic Organisms

For preserving binary cell division and endurance of microorganisms, low temperature is a precarious issue. Diminished synthesis and cold-denaturation of proteins, ice crystals formation, reduced cell membrane fluidity, lower rates of cross-membrane diffusion, and formation of highly reactive, harmful oxygen species are well-known serious consequences when microbes are exposed to low temperature. Microorganisms living in cold environments are typically exposed to a number of stress factors at the same time. For example, organisms living in the deep-sea usually need to sustain conditions of low temperature and high pressure in parallel, whereas microbes inhabiting Polar regions typically have to endure low temperature, nutrient shortage, and elevated UV-radiation in parallel. Accordingly, the search for microorganisms resistant to manifold stress factors in parallel leads to such extreme habitats (D'Amico et al. 2006). Various different sea-ice bacteria occupy small brine channels; such organisms are organized in "biofilms", which consist of a high number of cells and the extracellular polysaccharide excreted by them (Krembs et al. 2011). Restrictive substrate availability and low temperature act synergistically as growth-limiting elements; however, there are various reports on microorganisms, which are yet capable to thrive in cold water or even ice, unless both nutrient disposal and temperature drop below a critical level. Though sea ice is colder than the salt water beneath, the concentrations of substrates such as convertible carbon sources can be higher in the ice fraction; this enables microbes in marine ice to thrive, and, in some cases, also to accumulate PHA as carbon and energy storage compounds (Pomeroy and Wiebe 2001). Generally, presence of PHA inclusions increases the microbial viability in such challenging and strongly fluctuating environments.

Reports on PHA Production by Cryophilic Microorganisms

Pseudomonas sp. 14-3, a Gram-negative, rod-shaped, non-spore-forming, mobile bacterium from the Antarctic region was investigated by Ayub and colleagues (2009).

These authors have found out that the *phaRBAC* genes of this organism are placed on the same isolated region of the gene, where also those genes are located, which presumably contribute to its high adaptableness to low temperature. As follow-up, the impact of PHA biosynthesis on *Pseudomonas* sp. 14-3's adaptableness to low temperature was investigated by the same researchers, who directly compared the wild type organism and its PHA-negative mutant, in which the *phaC* gene encoding for PHA synthase was deleted. This mutant strain incapable of PHA accumulation turned out to be more prone to freezing than the wild type organisms; it was not able to grow at 10°C. The authors assumed that presence of PHA granules was essential to evolve adequate responses to the oxidative stress resulting from low temperature. It was further shown that the addition of reduced compounds, such as cysteine or gluthathione, contributes to suppressing the *phaC*-negative phenotype that features sensitivity against cold. Moreover, an abrupt cold shock incited prompt PHA degradation and strong decrease of both the NADPH content and the NADH/NAD ratio in the PHA-positive wild type organism. As a consequence, a 25-fold increase of lipid peroxidation was observed in the mutant strain at low temperature. Based on these outcomes, the authors concluded that the PHA metabolism triggers the disposal of the reducing equivalents NAD(P)H. Cold-related oxidative stress is relieved by degrading PHB, which generates the reduction potential needed to moderate the oxidative stress provoked by low temperature (Ayub et al. 2009).

Pseudomonas sp. 14-3 was later examined by López and colleagues by biochemical and physiological tests, and by 16S rRNA gene sequence analysis (López et al. 2009). By these characterization studies, it was demonstrated that this organism is a member of the genus *Pseudomonas sensu stricto*; it shows a gene sequence similarity of 99.7% to *Pseudomonas veronii* DSM 11331T, but DNA–DNA hybridization experiments with these two organisms showed rather low re-association similarity. Cultivation experiments with this bacterium revealed that the strain prospers well at temperatures between 4 to 37°C; however, at slightly thermophile conditions (42°C), no growth was observed. Using sodium octanoate as carbon source for this organism, the homopolyester PHB was produced. The authors suggested the classification of the strain as type strain of a new bacterial species called *Pseudomonas extremaustralis* sp. nov. Later, experiments with this organism and it's not PHA-producing mutant strain were performed by Tribelli and colleagues (2011). Using both planktonic cultures in shaking flask setups and immobilized cultures embedded in poly(styrene) biofilm microplates, these authors studied the connection between the strain's PHA- and EPS biosynthesis at low temperature. Motility, growth under microaerobic conditions, and PHA and EPS production were investigated. It was shown that, at low temperature, PHA biosynthesis occurs in parallel with higher cell motility; in addition, presence of PHA enhances the survival rate of planktonic cells. To some degree, microaerobic conditions preserved the deficit of the PHA-negative mutant for cold growth. The authors concluded that the PHB biosynthetic ability might be an adaptive benefit for microbes when occupying new

ecologically demanding niches. The entire genome of the strain was later sequenced by the authors in order to identify and categorize all genes involved in PHA biosynthesis and degradation, in flexibility towards extreme environmental conditions, and breakdown of harmful compounds such as diesel residues. These genomic studies resulted in the report of the first complete genome sequence of a cryophilic (psychrophilic) organism (Tribelli et al. 2011). The strain's bioremediation potential was investigated on the example of degradation of diesel, a harmful eco-contaminant. Such bioremediation processes typically resort to such microorganisms that can easily adapt to extreme surroundings, which are polluted with precarious compounds. To assess its bioremediation performance, *P. extremaustralis* was cultivated in agitated shaking flask setups (planktonic cells), or in benthic cultures, where cells were protected in biofilms. It turned out that cells embedded in biofilms, hence benthic cultures, grew better, produced more biosurfactants, and performed better in degradation of diesel than the planktonic cultures. PHA biosynthesis decreased the attachment of cells to the biofilm, and improved the formation of biosurfactants, as evidenced by surface tension tests. As a remarkable outcome, degradation of long-chain and branched alkanes occurred predominately by benthic in biofilms, whereas medium-chain length alkanes were mainly degraded by the action of free planktonic cells. Based on these outcomes, the authors concluded that the psychrophilic PHA-producing organism *P. extremaustralis* could be considered an auspicious candidate for bioremediation of diesel and other petro-fuels in extreme environments (Tribelli et al. 2012).

In the Antarctic Ecology Glacier foreland's freshwater reservoirs, Ciesielski and colleagues screened for further cryophilic microorganism able to accumulate PHA. The 16S rRNA gene sequence of the new isolates allowed classifying them as representatives of α-, β-, or γ-proteobacteria. This was followed by the PCR-based detection of the PHA synthase genes of each individual isolate. Organisms with evidenced potential for PHA biosynthesis were found at all sampling spots; the organisms were largely classified as members of the genera *Pseudomonas* and *Janthinobacterium*. This discovery of the regular occurrence of PHA producing organisms in such cold milieus was considered as another indication of PHA accumulation being a common metabolic characteristic of pioneering microorganisms. Those isolates which turned out to belong to the genus *Pseudomonas* displayed the genetic particularity to accumulate medium-chain-length PHA (*mcl*-PHA; this was shown by the presence of *phaC1* genes responsible for *mcl*-PHA biosynthesis), while those new strains belonging to the genus *Janthinobacterium* were able to produce either short-chain-length PHA (*scl*-PHA) or *mcl*-PHA (indicated by the detection of both *phaC* and *phaC1* genes). In this context, the study was the first at all reporting *mcl*-PHA production by members of the genus *Janthinobacterium*. Surprisingly, the authors also detected that, for the new *Janthinobacterium* sp. strains, no analogy can be found between the evolutionary history of the 16S rDNA genes and those genes that encode for the PHA synthases of these strains. *phaC* and *phaC1* genes of the

strains were phylogenetically analyzed; based on the outcomes, the authors suggested the entrance of the genomic material of *Janthinobacterium* sp. by horizontal gene transfer, and assumed that presence of these genes responsible for PHA biosynthesis may have evolved microbial survivability under environmentally extreme conditions (Ciesielski et al. 2014).

For a better understanding of the function of PHA biosynthesis in cold and highly saline ecological niches, Pärnänen et al. carried out a long-term study over a period of ten years. This study was dedicated to the search for PHA producing microbes in marine ice, in cold seawater, and in cold estuarine at the Swedish and Finish coast. Samples taken from these extreme environments were screened for PHA producers by Nile Blue A staining to trace PHA inclusions, and by PCR assays to identify genes encoding PHA synthase. Both PHA granules and PHA synthase genes were detected in all samples from the seawater and in ice samples. Based on *phaC* gene sequencing studies, high genetic homology of the isolates was evidenced with the β-proteobacterial strains *Rhodoferax ferrireducens*, *Polaromonas naphthalenivorans* and *Polaromonas* sp., and the γ-proteobacterium *Pseudomonas extremaustralis* strain 14-3. The outcomes of this study revealed a significant biodiversity of the cold and highly saline region of the Baltic Sea, especially the tremendous wealth of microbes adapted to the environmental conditions, which makes this remote area an interesting pool for the search for new PHA production strains (Pärnänen et al. 2015).

Obruca and associates resorted to cryo scanning electron microscopy (cryo-SEM) to mechanistically describe the cryo-protective effect of PHB in bacterial survivability under freezing conditions (Obruca et al. 2016a). Cryo-SEM constitutes a modern tool, which enables investigating the impact of PHA inclusion on the mechanical properties of the cell, on solutes' mobility in the cytoplasm, and the cellular response to sudden temperature changes such as freezing, towing, or drying (Kryzyzanek et al. 2015). In 2017, Dubochet, Frank, and Henderson were awarded the Nobel Prize in Chemistry for the development of this groundbreaking technique, which even enables the recognition of structures of biomolecules in solution. The authors found out that, during freezing-and-thawing cycles, 3HB, the monomer of PHB, reveals a strong cryo-protective effect for lipase, yeasts, and *C. necator* as an important PHB production strain. When comparing the survival rates of PHB-positive *C. necator* cultures with cultures of the PHB-negative mutant strain during freezing-and-thawing cycles, it was shown that viability was significantly increased in the PHA-positive cultures. Hence, the presence of PHB inclusions provided the cells with a significant survival advantage during freezing. The typical high levels of 3HB in such cells that are able to simultaneously produce and degrade PHB, such as *C. necator*, was suggested by the authors to be mainly responsible for this protective effect. However, this study also revealed that the cryo-protective mechanisms of PHA granules seem to be more complex and multi-facetted. As observed by cryo-SEM observations, PHA inclusions maintain their outstanding flexibility still at

exceptionally low temperatures; this suggests that the PHA granules inhibit the occurrence of cellular destruction caused by the intracellular formation of ice crystals. Typical cell damages caused by ice crystal formation encompass the formation of gas bubbles, physical membrane damage, or even destruction of organelles. Mechanistically, it seems like the presence of PHA inclusions alters the adhesion forces between cellular components and water molecules; due to a higher transmembrane water transport rate, water in cells containing PHA granules can be more rapidly discharged from the cell during drying or freezing, thus preventing ice formation (Obruca et al. 2016a).

THERMOPHILIC PHA PRODUCERS

General Features of Thermophiles

At first glance, biotechnological use of thermophiles seems to be problematic due to some technological problems associated with cultivation at high temperature such as decreased solubility of oxygen at elevated temperatures and especially energetic demands to maintain high cultivation temperature. Nevertheless, when thermophilic micro-organisms are used in bioreactor cultivations, these processes are, surprisingly, regarded being of higher energy efficiency, mainly because minor efforts for energy-demanding cooling are needed. Moreover, such thermophilic cultivations are "self-heating systems" because of the heat energy generated by the microbial metabolism, a phenomenon, which is particularly observed at cultivation batches which are carried out with high cell concentration in large scale cultivations. Further, also the stirring system of the bioreactor generates heat energy, which can additionally be used for the bioprocess. Combining these manifold effects allows reducing of both heating and cooling expenses. As a further advantage in comparison to the application of thermo-mesophilic microbes, also sterility precautions typically needing heating can be minimized when thermophilic organisms are used; this advantage is analogous to the energetic benefit described above for the use of halophiles (Ibrahim and Steinbüchel 2010).

Recently, Obruca and associates made mechanistic reflections about how high temperature and the metabolic PHA cycle are interrelated (Obruca et al. 2016b). Based on these considerations, the authors reported the function of the monomer 3HB as a chemical chaperone, which prevents the denaturation of a range of enzymes such as lipase and lysozyme at high temperature and strongly oxidative conditions. In this study, lipase denaturation caused by high temperature was investigated both in the presence and absence of 3HB; as result, a strong protective effect of 3HB as product of the functional PHB metabolism was shown. This effect was even more pronounced than in the case of well-described chemical chaperones, e.g., hydroxyectoine or trehalose. The monomer of PHB successfully protected lipase not only against denaturation caused by high

temperature; similarly, the protective role of 3HB against oxidative damage caused by Cu^{2+} and H_2O_2 was demonstrated. In this context, the protective effect was higher than described for trehalose, and in the same range as known for hydroxyectoine. These outcomes approve preceding studies accomplished by Soto and colleagues, who already proposed that PHA monomers play a role in preventing enzyme denaturation under conditions of high temperature and extreme salinity (Soto et al. 2012).

Gram-Negative Thermophile PHA Producers

PHA production by the thermophilic Gram-negative bacterium *Thermus thermophilus* was for the first time investigated by Pantazaki and coworkers (2003). These researchers carried out experiments at extremely high temperature conditions of up to 75°C, using sodium gluconate or sodium octanoate, respectively, as the sole carbon sources. PHA mass fractions in CDM of about 35–40 wt.-% were achieved in these shaking flask cultivation setups. Dependent on sodium gluconate or sodium octanoate, respectively, being used as carbon source, completely different types of PHA regarding their monomeric composition were obtained. While a copolyester consisting of about 64 mol-% of 3-hydroxydecanoate (3HD), and minor 3-hydroxyoctanoate (3HO), 3HV, and 3HB fractions was obtained when gluconate was used, the biopolyester produced by octanoate-based cultivation was composed as follows: 24.5 mol-% 3HB, 5.4 mol-% 3HO, 12.3 mol-% 3-hydroxynonanoate (3HN), 14.6 mol-% 3HD, 35.4 mol-% 3-hydroxyundecanoate (3HUD), and 7.8 mol-% 3-hydroxydodecanoate (3HDD). The weight average molecular mass (M_w) of the gluconate-based copolyester amounted to 480,000 g/mol, whereas a M_w of 391,000 g/mol was reported for the octanoate-based product. In the soluble cytosolic fraction obtained from the gluconate-based *T. thermophilus* cells cultivation, the activities of 3-ketothiolase, NADPH-dependent reductase, and PHA synthase, hence, the enzymes catalyzing PHA biosynthesis, were determined. PHA synthase obtained from these gluconate-grown cells was isolated, purified, and subjected towards characterization. The optimum values for temperature and pH-value of these enzyme amounted to about 70°C and a pH-value of 7.3, respectively. Further, the supplementation of free CoA and alkaline phosphatase entirely inhibited the catalytic activity of this enzyme. In contrast to other described PHA synthases, there was no lag phase visible when monitoring the enzyme kinetics of *T. thermophiles* PHA synthase. Moreover, the authors assumed an essential function of cysteine in the enzyme's biocatalytic mechanisms; in any case, this enzyme isolated from *T. thermophilus* constitutes the first characterized PHA synthase isolated from a thermophilic organisms (Pantazaki et al. 2003). In a subsequent study, the same group of authors identified all genes responsible for PHA biosynthesis in the genome of *T. thermophilus* by resorting to PCR techniques. These genetic studies confirmed that the

PHA synthase of the strain *T. thermophilus* HB8 belongs to the Class-II PHA synthases, as typically found in *mcl*-PHA producing Pseudomonades (Papi et al. 2008).

Thermophile PHA producers are also found among phototrophic organisms. In this context, Hai et al. (2002) tested eleven different cyanobacterial strains for their PHA production potential, and characterized the type of PHA synthase catalyzing PHA synthesis by the individual strains. For these characterization studies, Southern blot analysis with a *phaC*-specific DNA probe, PCR sequence analysis, Western blot analysis with specific polyclonal anti-*phaE* antibodies complementary to *Synechocystis* sp. strain PCC 6803 *PhaE*, and PHA synthase structural gene sequence analysis were applied. Outcomes of the applied methods confirmed the presence of Class-III PHA synthases in the cyanobacterial strains *Anabaena cylindrica* SAG 1403-2, *Chlorogloeopsis fritschii* PCC 6912, different *Cyanothece* sp. (strains PCC 7424, PCC 8303 and PCC 8801), *Gloeocapsa* sp. strain PCC 7428, and *Synechococcus* sp. (strains MA19 and PCC 6715). Contrariwise, Class-III synthase genes were not found in the cyanobacterial strains *Cyanothece* sp. PCC 8955, *Gloeothece* sp. PCC 6501, and *Stanieria* sp. PCC 7437. *Synechocystis* sp. strain PCC 6803, the first cyanobacterial strain with entirely characterized PHA synthase genes, was used as positive control by comparing it with protein extracts and chromosomal DNA of the investigated strains. Later, all *Chlorogloeopsis fritschii* PCC 6912 and *Synechococcus* sp. MA19 PHA synthase structural genes, and a central region of the *phaC* gene from *Cyanothece* sp. PCC 8303 were cloned, sequenced, and transferred into *Escherichia coli* in order to be heterologously expressed (Hai et al. 2002).

The thermophile cyanobacterium *Synechococcus* sp. MA19 is known for photoautotrophic production of PHB at high quantities. Miyake and colleagues (1996) first described the isolation of this organism from a Japanese volcano rock. 50°C was identified as the optimum temperature to thrive this organism photoautotrophically in simple cultivation bottles. These bottles were aerated by a submerged airstream enriched with 2% CO_2. It was shown that the organism is able to accumulate a PHA mass fraction in CDM of up to 21 wt.-% PHB. Based on this study, the authors suggested *Synechococcus* sp. MA19 as the first cyanobacterium that might be an auspicious candidate for future PHA production on an industrially relevant scale. Optimization of the CO_2 supply and the illumination regime (wavelength, intensity, etc.) are key factors for improving PHA productivity by this organism. For large-scale implementation, this requires optimized process engineering. When cultivation setups were carried out without illumination and nitrogen-deficiency, the PHB mass fraction in CDM increased to even 27 wt.-%. This increase originated from the degradation of glycogen, the second reserve material stored in *Synechococcus* sp. MA19, in the dark. This is in contrast to the PHB reserves, which are only degraded by this organism under illumination and parallel nitrogen availability; PHB degradation was not observed in the dark by this strain, neither under nitrogen-rich nor nitrogen-poor conditions (Miyake et al. 1996).

Nishioka and colleagues (2001) cultivated thermophile cyanobacteria under conditions of phosphate- instead of nitrogen limitation in order to boost PHA productivity. At high temperature of 50°C, and under autotrophic and phosphate limited conditions, *Spirulina* sp. MA19 was cultivated at intracellular phosphate concentrations of 0.043 to 0.076 mmol per g CDM. After 260 hours of cultivation using the hardly water soluble phosphate source $Ca_3(PO_4)_2$, a PHB mass fraction in CDM of 55 wt.-%, a PHB concentration of 2.4 g/L, and 4.4 g/L CDM were obtained. This was almost the double productivity if compared with cultivation batches with sufficient phosphate supply. Based on these outcomes, it was proposed by the authors to design fed-batch cultivation of *Spirulina* sp. MA19 in a way avoiding intracellular phosphate accumulation by carefully supplying the phosphate source (Nishioka et al. 2001).

Also chemoorganotrophic thermophilic PHA producers are described in literature. In this context, an amylase-producing organism was isolated in Pingtung, Taiwan, by Chen and associates from a hot spring (2005). This isolate was labeled as strain On1T. The organisms was investigated and turned out to be Gram-negative, forming rods of monotrich flagellation. Around 55°C and a neutral pH-value were shown to be the optimum temperature- and pH-range, respectively, to thrive this strain. Using 16S rRNA gene sequence analysis for phylogenetic classification, the organism was identified as a member of the β-proteobacteria class. By the sum of results from physiological and biochemical tests, fatty acid pattern analysis, 16S rRNA sequence analysis, and DNA–DNA similarity tests, it was shown that the strain displays a new species of the genus *Caldimonas*; *Caldimonas taiwanensis* sp. nov. was suggested by the researchers as strain name for this new thermophile isolate (Chen et al. 2005). Later, Sheu et al. revealed that *C. taiwanensis* accumulates PHB at 55°C from a range of frequently used carbon sources such as glycerol, fructose, maltose, and gluconate, when nitrogen source availability is restricted. However, no PHB accumulation was monitored when using fatty acids as sole carbon source. By co-feeding a substrate mixture of gluconate and the 3HV-precursor valerate, the copolyester PHBHV was accumulated instead of the homopolyester PHB. In dependence on the valerate concentration and the valerate-to-gluconate ratio in the medium, it was possible to adjust the molar 3HV fraction in PHBHV in a range between 10 and 95 mol-% (Sheu et al. 2009). These outcomes are similar to those obtained before using the strain *Caldimonas manganoxidans*, another PHA producer with a temperature optimum also in the range of about 50°C by (Takeda et al. 2002). In the *C. taiwanensis* experiments carried out by Sheu et al., presence of valerate considerably inhibited both growth and PHA accumulation at concentrations as low as 5 mM. This is similar to valerate's inhibitory effect on other PHA producing strains such as, e.g., *Burkholderia funghorum* (previously known as *Pseudomonas hydrogenovora*) (Koller et al. 2008b). In the case of *C. taiwanensis*, it was shown that this inhibitory effect could be reduced when supplementing yeast extract; this enhanced both the biomass yield and PHA accumulation (Sheu et al. 2009). Later, *C. taiwanensis* PHA synthase was isolated and

investigated in *in vivo* tests. It turned out that the isolated enzyme is specific for substrates 3HB, 3HV, and the *mcl*-PHA building block 3-hydroxyhexanoate (3HHx). Due to the amylase activity of the strain, cultivations were carried out on mixtures of soluble cassava starch, corn starch, potato starch, sweet potato starch, and wheat starch as main substrates and valerate as 3HV-related precursor compound. Also these cultivations resulted in the production of PHBHV copolyesters; dependent on the starch-to-valerate ratio, the copolyesters contained different molar 3HV fractions. All over, the authors emphasized the high potential of *C. taiwanensis* as candidate strain for cost-efficient production of PHBHV; this is due to the strain's ability of converting inexpensive starch of different origin as sole carbon source, and the minimized cooling costs, which originate from the thermophile process regime (Sheu et al. 2009).

A considerable number of additional thermophile microorganisms, which are able to utilize inexpensive materials as carbon sources for PHA biosynthesis at high temperature were later isolated and assessed by Ibrahim and colleagues (2010). On the one hand, an aerobic waste treatment plant in Germany, and, on the other hand, hot springs in Egypt were the locations were samples for screening of microbes were taken. Colonies were cultivated on solid agar plates with different defined carbon sources and Nile Red in order to spot PHA-accumulating colonies. Six Gram-negative bacteria were identified by this screening. Among these isolates, five strains, labeled MW9, MW11, MW12, MW13 and MW14, formed stable star-shaped cell aggregates (SSCAs) during growth, whereas the isolate MW10 appeared in the microscope as non-aggregated planktonic rods. All six isolates belonged to the class of α-proteobacteria, as shown by16S rRNA gene sequence analysis. This 16S rRNA gene sequence analysis revealed that all these isolates have more than 99% gene sequence similarity to each other, and, comparing to other known strains, highest sequence similarities of 93 to 99% with species belonging to the genera *Bosea*, *Chelatococcus*, and *Methylobacterium*. Glucose turned out as the preferred carbon source of the isolates MW9, MW10, MW13 and MW14, while best growth and highest PHB production for MW11 and MW12 occurred when cultivating these strains on glycerol as sole carbon source. Maximum values obtained by the isolates for CDM concentration and PHB mass fraction in CDM amounted to 4.8 g/L and 73 wt.-%, respectively. All these organisms displayed growth in a temperature range between 37 and 55°C, with 50°C being the optimum temperature for growth. The authors emphasized that this study constitutes the very first report on SSCA-forming thermophile and PHA-accumulating organisms; further, they underlined again that using thermophiles for PHA production provides a high potential to make this process economically feasible. 16S rRNA gene sequence analysis of the isolate MW9 revealed its close relationship to the genus *Chelatococcus*. More detailed experiments were later carried out for a better classification of the isolates by fatty acid pattern analysis, rep-PCR genomic fingerprints, G+C content analysis in DNA, and partial *dnaK* gene sequencing; these tests confirmed

the strain's close relationship of MW9 and other isolated *C. taiwanensis* ssp.; however, the tests also suggested that some differences exists to other described members of *Chelatococcus*. Hybridization of the DNA of MW9 or other isolates with the DNA of other *Chelatococcus* sp. exposed degrees of relationship of only 11.0 to 47.7%. Therefore, the authors proposed classifying the isolate MW9 as a novel *Chelatococcus* sp., and suggested the name *Chelatococcus thermostellatus* for this auspicious candidate for future industrial PHA production at high temperature (Ibrahim et al. 2010).

Chelatococcus sp. strain MW10 is another isolate of the strain screening series of Ibrahim. This strain was cultivated by these researchers by advanced cultivation strategies in order to obtain higher CDM concentrations. On a 2 L bioreactor scale, fed-batch fermentation processes were carried out using pulse feeding of glucose as sole carbon source to keep it's concentration above 20 g/L. This excess glucose feeding approach was selected to increase both residual biomass and PHB synthesis, and to prevent intracellular PHA from degradation. After 53 h of cultivation, a maximum CDM concentration of 5.2 ± 0.6 g/L was obtained; the maximum PHB concentration amounted to 2.9 ± 0.7 g/L. Remarkably, despite the permanent glucose availability, the intracellular PHB content significantly decreased in the later cultivation phase. To overcome this shortcoming, a cyclic batch fermentation (CBF) strategy was tested; this was accomplished in a 42 L bioreactor at 50°C and a high carbon source level of 50 g/L glucose. The cycling time was fixed with 50 h, which is in accordance with the optimum outcomes of the cultivations on 2 L scale. The fermentation was started with an initial volume of 25 L; by adapting the agitation speed of the stirrer system and the aeration rate, pO_2 was kept constant at 20% of air saturation. A high growth rate of $\mu_{max.}$ = 0.125 1/h, and considerably higher CDM concentration of up to 12.7 ± 0.9 g/L were obtained in the first 50 h cycle; however, PHB mass fraction in biomass amounted to only 55.0 ± 5.7 wt.-%, which is in the same range as it was obtained in the 2 L fed-batch setups. After this first cycle, a considerable part of the fermentation broth, namely 23 L, were withdrawn, and 23 L of fresh, non-sterilized nutrient medium were added to the remaining cultivation broth, which was used as a strong inoculum to start the subsequent fermentation cycle. This second cycle delivered comparable CDM concentration (about 11 g/L), but, despite the permanent availability of carbon source, decreased intracellular PHB mass fractions in CDM of only 38.5 ± 6.4 wt.-%; in a third cycle performed the same way as described for the second one, the PHB fraction in biomass amounted to only 32.5 ± 3.0 wt.-%. As additional process development, a modified semi-continuous cultivation strategy, the so-called cyclic fed-batch fermentation (CFBF), was tested on 42 L scale. This cultivation mode was characterized by replacement of different volumes of the fermentation broth by fresh medium, which results in a partially recycling of 20 to 40% of the volume of the fermentation broth. The culture was cycled at irregular intervals as response to volume increase because of the semi-continuous feeding, and to

the decreased pO_2 level occurring due to the high cell density in this fed batch process. Generally, proper glucose (carbon source) and ammonium (nitrogen source) concentrations were used to optimize CDM concentration and the intracellular PHB mass fraction. Glucose at 30 g/L was used to start the CFBF process in batch mode. After 21 h, fresh medium was fed for the first time. The first cycling, accomplished by replacing 5 L of fermentation broth by fresh medium, occurred after 44 h of cultivation, hence, at a time when specific growth of cells was relatively high ($\mu_{max.}$ = 0.070 1/h) and PHB degradation was not observed yet. After this first cycling, feeding occurred continuously, and further medium replacement cycles were accomplished according to excessive volume increase in the bioreactor. To prevent the cultivation broth from getting excessively diluted by fresh medium, 10 L of culture volume were withdrawn to complete the second cycle, and replaced by fresh medium only half of this volume (5 L). When the third cycle was operated for 14 h, a last refilling of 5 L of fresh medium was done. For the period between 82 h and 143 h in the second cycle, the highest PHB mass fractions exceeding 50 wt.-% were reported. After 181 h, this cycle was finished. At that time, CDM amounted to 43.0 ± 1.4 g/L CDM, the intracellular PHA fraction in CDM was determined with 39.0 ± 8.5 wt.-%, and the maximal PHB concentration reached 16.8 ± 4.2 g/L. Cell growth was significantly boosted until the end of the third cycle; here, CDM reached 115.0 ± 4.3 g/L. Despite the fact that, at the end of this process, a lower intracellular PHB fraction of 11.8 ± 3.8 wt.-% was obtained, the volumetric PHB productivity of 13.7 ± 4.9 g/L displays a promising result (Ibrahim et al. 2010).

In 2014, Xu and colleagues isolated the thermophilic organism *Chelatococcus daeguensis* TAD1, a species closely related to Ibrahim's above described *Chelatococcus* sp. strain MW10. The habitat were the sample derived from was the biological layer of a bio-trickling filter used for NO_x removal. *Chelatococcus daeguensis* TAD1, a thermophilic PHB-producing bacterium, was comprehensively studied and compared to other PHA-accumulating bacteria. Without limitation of growth-essential nutrients, the strain produced PHA in parallel to microbial growth. At elevated temperature of 45°C, *Chelatococcus daeguensis* TAD1 stored an intracellular PHB fraction in CDM of up to 83.6 wt.-% when glucose was used as the sole carbon source in a 24 h cultivation process. It is noteworthy that the strain was highly tolerant both to stress provoked by heat and to the high nitrogen levels present during PHB biosynthesis. Glucose as carbon source, a cultivation temperature of 50°C, and a molar carbon-to-nitrogen-ratio of 1/30 were identified as those process conditions resulting in the highest PHA concentration of 3.44 ± 0.3 g/L. As additional benefit, the strain was able to efficiently utilize a variety of inexpensive carbon sources for PHA production, among them non-hydrolyzed starch or glycerol. On glycerol, the highest PHB yield and the highest intracellular PHB fraction where obtained with values of 0.26 g PHB per gram glycerol, and 80.4 wt.-%, respectively. Consequently, the authors suggested *C. daeguensis* TAD1 as another

auspicious cellular factory, applicable for production of PHA from abundantly available substrates on an industrial scale (Xu et al. 2014). PHA production performance of this strain was further improved by the same group of researchers, who applied a fed batch-feeding regime for PHB production by *C. daeguensis* TAD1 on glycerol. The authors revealed the inhibitory effect of excessive glycerol concentrations on PHB biosynthesis by this strain, but, at the same time, discovered that addition of complex nitrogen sources such as yeast extract or tryptone is highly beneficial to increase biomass formation of this organism. Consequently, batch fermentations of *C. daeguensis* TAD1 were carried out using low glycerol concentrations, and supplying a nitrogen-rich substrate cocktail containing yeast extract, tryptone, and NH_4Cl. In these batch-setups, PHB production by *C. daeguensis* TAD1 was considerably boosted. Based on the results of these batch-results, further cultivation setups were carried out in fed-batch and two-stage fed-batch cultivation mode. Best results for PHB concentration were obtained in the two-stage fed batch setup with 17.4 g/L, which corresponds to a volumetric productivity of 0.434 g/(L·h). This value constitutes the up to date highest reported PHA productivity when using extremophilic microbes (Cui et al. 2015).

A report delivered by Romano and associates describes a haloarchaeon, which is both thermophilic and extremely halophilic. This organism, isolated from the Fuente de Piedra salt lake in southern Spain, was identified by the authors as the new species *Haloterrigena hispanica* sp. nov. This haloarchaeon thrives best in a temperature range between 37°C and 60°C with a growth optimum at 50°C, and requires at least 150 g/L NaCl for growth. Even 200 g/L NaCl turned out as beneficial for cultivation of this strain, hence, the optimum salinity of this organism is identical to the requirements of *Hfx. mediterranei*. In contrast to other described halophile PHA producers, *H. hispanica* sp. nov. does not require Mg^{2+} ions for growth. According to the authors, the strain accumulates the homopolyester PHB; unfortunately, no detailed data on PHB productivity were provided in this publication (Romano et al. 2007).

In a later study carried out by Mastascusa and colleagues, *H. hispanica* was used as one out of four extremophile model organisms, which were exposed to extreme conditions of temperature, UV radiation, and desiccation coupled to low atmospheric pressure. These extreme conditions were applied to simulate conditions on the planet Mars, hence, the study contributed to the current quest for extraterrestrial life forms (Mastascusa et al. 2014). In this context, haloarchaea are of special interest as organisms, which potentially could survive and prosper on Mars; this is due to the fact that the pressure in the Martian atmosphere is below water's triple point, which makes it impossible for fresh water organisms to survive. All investigated extremophiles, namely *Geobacillus thermantarcticus*, *H. hispanica*, *Sulfolobus solfataricus*, and *Thermotoga neapolitana* displayed good resistance to the simulated Mars temperature variations; *G.*

thermantarcticus, *H. hispanica*, and *S. solfataricus* proved to be highly resistant to UV-irradiation at 254 nm, whereas *G. thermantarcticus* and *H. hispanica* outperformed the other strains when exposed to desiccation and low pressure (Mastascusa et al. 2014).

Gram-Positive Thermophile PHA Producers

Generally, considering the vast number of Gram-negative bacteria and Archaea which are described as PHA producers, only an evanescent number of efficient Gram-positive microbial PHA producers is reported, particularly considering the tiny number of extremophiles among Gram-positive PHA producers (Rodriguez-Contreras et al. 2013b). As already mentioned in the general part of this chapter, PHA biopolyesters synthesized by Gram-positive microbes are not associated with immunogenically active LPS, thus making these materials more suitable for *in vivo* application. Liu et al. delivered the first report on a thermophilic Gram-positive bacterium able to produce PHA. These authors described PHA biosynthesis by the bacterial isolate K5, a strain displaying excellent performance regarding growth kinetics and PHB storage capacity. Similar to *Chelatococcus daeguensis* TAD1, strain K5 originates from the biomat of a biotrickling filter, which was used in a coal-fired power plant in China to mitigate NO_x from effluent gas. Physiological and biochemical characterization and 16S rRNA gene sequence analysis was carried out to better characterize this organisms; based on these tests, it became clear that strain K5 has to be classified as subspecies of *Bacillus shackletonii*, a species hardly described at all in literature. *B. shackletonii* K5 utilized glucose as carbon source for PHB synthesis in a rather broad temperature range between 35 to 50°C, with the optimum temperature for growth being 45°C. In shaking flask scale experiments on glucose, *B. shackletonii* K5 accumulated an intracellular PHA mass fraction of up to 69.9 wt.-% PHB; CDM amounted to 2.28 g/L. When succinate or glycerol were used as carbon sources, the biopolyester fractions in CDM amounted to 56.8 and 52.3 wt.-%, respectively. Later, batch-mode cultivations on glucose were carried out under controlled conditions at a temperature of 45°C and a pH-value of 7.0; in these setups, PHB concentration and PHB mass fraction in CDM amounted to 9.76 g/L and 72.6 wt.-%, respectively (Liu et al. 2014).

Xiao and associates performed additional isolation studies to screen new thermophilic, robust PHA production strains. From oilfield samples, the organism XH2 was isolated in this screening study. This strain was characterized morphologically, physiologically, biochemically and by 16S rDNA sequence analysis. As a result, the strain was classified as *Aneurinibacillus* sp. PHA granule accumulation by this organism was evidenced by Nile red staining and transmission electron microscopy (TEM) technique. This study was the first confirming the presence of PHA inclusions in a

thermophilic member of the phylum *Firmicutes*. Batch cultivations were carried out with this organism at 55 °C using glucose, yeast extract, and peptone, resulting in the rather modest production of 0.12 g/L PHA. This product concentration was increased more than two-fold when using peptone-free medium. The obtained PHA biopolyester was mainly composed of the *scl*-PHA building blocks 3HB and 3HV; in addition, the occurrence of smaller quantities of 3HO and the aromatic monomer 3-hydroxy-4-phenylbutanoate was evidenced via NMR and GC-MS (Xiao et al. 2015).

Geobacillus sp. AY 946034 is another thermophilic organism capable for PHB biosynthesis, which was isolated from a Lithuanian oil field by Giedraitytė and colleagues. This organism exhibited positive results when subjected towards Sudan Black staining. It thrives well on various carbon sources such as acetate, glucose, maltose, sucrose, lactose, glycerol, and starch at temperatures as high as up to 60°C. In shaking flask setups using a mineral medium and glucose as the most promising carbon source together with phosphate deprivation, which turned out to better boost PHB biosynthesis than nitrogen limitation, the strain accumulated PHB up to a mass fraction in CDM of 68.9 wt.-%. Remarkably, no PHB biosynthesis was observed using acetate or glycerol. It was assumed by the authors that *Geobacillus* sp. AY 946034 resorts to the same well-known PHA biosynthesis pathway as described for other *scl*-PHA producers; this assumption was based on the detection of high activities of the major PHA biosynthetic enzymes 3-ketothiolase, NADH-dependent acetoacetyl-CoA reductase, and PHA synthase. The PHB produced by the strain was isolated by boiling chloroform, and subjected towards chemical characterization via FTIR, thermoanalytical analysis via DSC, and molecular mass determination by using viscosity measurement. Outcomes of the FTIR analysis confirmed 3HB as the only monomeric constituent of this homopolyester, which displayed semi-crystalline characteristics with a degree of crystallinity X_c of 42%, a melting temperature T_m of 169°C, a T_d of 280°C, and a melting enthalpy (H_m) of about 63 J/g as revealed by DSC analysis. By molecular mass analysis, M_w was determined with 556 kDa (Giedraitytė et al. 2015).

Thermostable enzymes are needed on order to perform efficient and fast *in vitro* PHA synthesis. Such *in vitro* synthesis of PHA was reported by Tajima et al., who used an engineered thermostable PHA synthase labeled PhaC1SG(STQK). However, this engineered system contained also enzymes from mesophilic organisms; this addresses the application of not thermostable acetyl-CoA synthetase (ACS) and acetoacetyl-CoA transferase (CT), which are needed for generation of the monomers. In order to develop an entirely thermostable *in vitro* system for PHA synthesis at high temperature, such enzymes were cloned from thermophiles, namely ACS from *Pelotomaculum thermopropionicum* JCM10971 and CT from *Thermus thermophilus* JCM10941. Both ACS and CT turned out to be highly thermostable, with temperature optima determined at around 60°C for ACS, and 75°C for CT, respectively. An *in vitro* PHB and poly(lactate-*co*-3HB) synthesis enzyme cascade was successfully tested at 45°C by the concerted

biocatalytic action of *Thermus thermophilus* ACS, *Thermus thermophilus* CT, and the synthase PhaC1SG(STQK). Further experiments with this enzyme cascade were carried out at moderate temperature (37°C); also under these thermo-mesophilic conditions, the yields for both PHB and poly(lactate-*co*-3HB) biosynthesis by this thermophilic enzymes were still superior than results previously obtained by the authors when using non-thermostable ACS and CT (Tajima et al. 2016).

An economic study was recently developed by Levett and colleagues, which emphasizes that the lack of amply accessible, inexpensive feedstocks constitutes the main hurdle on the way towards PHA production on a large scale (Levett et al. 2016). The low market price und abundant availability of methane makes this gas an auspicious carbon source for PHA production at industrially relevant scale (Khosravi-Darani et al. 2013). These authors developed an all-inclusive techno-economic analysis of PHA production based on the utilization of methane as sole carbon source. This analysis was carried out software-aided by using ASPEN Plus for designing the engineering, modelling, and simulation. The simulation was based on an assumed annual PHB production scale of 100,000 t PHB considering methanotrophic cultivation in a two-stage bioreactor process, followed by PHB extraction based on an acetone-and-water system. An assessment of the economics of the process suggests a tentative production price of US-$ 4.1–6.8 per kg PHA; this price is similar to prices for PHA production on other carbon sources, as reported by other studies (Levett et al. 2016). Whereas for sucrose-based PHA production processes, the costs of the carbon source account by about 30-50% to the entire production costs (Nonato et al. 2001), this share decreases to only 22% for the calculated methane-based process (Levett et al. 2016). As another important aspect, the authors calculated that about 28% of the entire plant operation costs are allotted to the removal of heat from the bioreactors. For this reason, the use of thermophilic methanotroph production strains could considerably contribute to cost reduction; specifically, the authors suggested using cooling water instead of expensive refrigeration compounds, which additionally decreases the production price per kg PHA to US-$ 3.2–5.4 (Levett et al. 2016); this value is already in a similar range as calculated for whey-based PHA production by haloarchaea (Koller et al. 2007a). Also costs for the energy needed for air compression and biomass drying before PHA recovery were identified as factors considerably contributing to the overall process expenses. These costs could be reduced by optimizing the bioreactor's geometry, the process conditions, and techniques for biomass drying. In addition, a detailed comprehension of the methane mass transfer at high cell density and elevated pressure, and minimized acetone loss during the PHA extraction process are required for further improvement of cost-efficiency. Nevertheless, it should be underlined that these estimations are based on *in silico* calculations based on a fictive thermophile model production strain. To assess the validity and robustness of these simulations, reliable laboratory results for PHB biosynthesis by thermophilic methanotrophs are needed.

ADDITIONAL STRESSORS BOOSTING PHA BIOSYNTHESIS

PHA accumulation generally supports viability, stress resistance and cell robustness of microbes under various environmentally unfavorable conditions. It is probably the reason why so many extremophiles (as was demonstrated above in the text) possess the capability of PHA accumulation. The stress protective mechanism of PHA is very complex and involves numerous metabolic but also biophysical consequences of presence of PHA granules in bacterial cells (reviewed by Obruca et al. 2018). For instance, PHA granules associated protein PhaP was recently identified as efficient chaperone preventing proteins from losing their native structures (Mezzina et al. 2015). Moreover, chaperoning activity was reported also for PHA monomers which are released from PHA during constant depolymerization of PHA (Soto et al. 2012, Obruca et al. 2016b). Moreover, some PHA accumulating bacteria are able to convert PHB into methyl-esterified dimers and trimers which protect bacteria from hydroxyl radicals more efficiently than glutathione or vitamin C (Koskimaki et al. 2016). Functional PHA metabolism helps cells to maintain optimal redox potential under unfavorable circumstances (Ayub et al. 2009) and is associated with a stationary phase regulator which activates the expression of genes providing cross-protection against multiple stressors (Ruiz et al. 2001). Moreover, biophysical consequences of presence of PHA granules in cell cytoplasm protects bacteria from damaging UV radiation and disruption of cell integrity and membrane damage under hypertonic conditions and during freezing and subsequent thawing (Obruca et al. 2016a, Obruca et al. 2017).

The fact that PHA biosynthesis is interconnected with stress response is not only of fundamental interest but it can also have practical outcome since induction of controlled stress conditions can be used as a tool to trigger PHA accumulation in bacteria during PHA production. For instance, it was reported that exposure of *C. necator* to mild levels of some stressors such as NaCl, ethanol or H_2O_2 at the beginning of the stationary cultivation phase boosts PHB accumulation by about 30% (Obruca et al. 2010b). The positive effect of ethanol and hydrogen peroxide was further investigated and it was supposed that the pentose phosphate pathway is accelerated by the presence of H_2O_2, which consequently results in a higher ratio of NADPH/$NADP^+$, hence, in an increased intracellular concentration of reducing equivalents. This increase of reducing equivalents in turn causes a slight inhibition of the citric acid cycle (TCA), which shifts the carbon flux in the form of acetyl-CoA towards PHB accumulation, and increases the enzymatic activities of the enzymes 3-ketothiolase and acetoacetyl-CoA reductase, without at the same time effecting the activity of PHA synthase. Also the mechanistic function of ethanol addition for increased PHA biosynthesis was described to derive from the increased pool of the reducing equivalents NAD(P)H, which is accumulated because of the alcohol dehydrogenase-catalyzed oxidation of ethanol. In addition to PHA hyper-production triggered by these stress factors, molecular mass of the biopolyesters was

considerably higher when cells were exposed to the stressors H_2O_2 and ethanol than it was the case in control cultivations carried out under balanced conditions. Data for molecular mass were strongly dependent on the concentration of the stress factors. The authors assumed that the exposure of *C. necator* cells to exogenous stress factors such as strong oxidants or organic solvents could be considered a simple approach to considerably enhance PHB biosynthesis by this strain (Obruca et al. 2010a). The positive effect of appropriate oxidative pressure on PHA biosynthesis was further confirmed in another study in which bacterial cells of *C. necator* were exposed to random mutagenesis; here, the PHA hyper-producing mutant revealed substantially enhanced activities of NADPH generating enzymes such as malic enzyme, NADPH-dependent isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and glutamate dehydrogenase. Hence, the mutant demonstrated oxidative stress adapted metabolism features, which resulted in enhanced intracellular $NADPH/NADP^+$ ratio supporting PHA accumulation. Furthermore, the mutant was also able to incorporate propionate into the copolymer PHBHV more efficiently than observed for the wild-type strain (Obruca et al. 2013).

Moreover, also alcohol-induced PHA hyper-production has practical bio-technological use, since another work dealing with PHA production employing *C. necator* H16 growing on waste rapeseed oil as main carbon source for PHA accumulation reported that media supplementation with different alcohols (ethanol, methanol, and 1-propanol) that normally constitute cellular toxins for many organisms can enhance PHA production. Among these alcohols, especially supplementing 1-propanol to 24 h fed-batch cultures of *C. necator* H16 increased biomass growth from around 11 to around 15 g/L CDM, and also PHA formation increased from about 6 g/L in control experiments to about 12 g/L when supplying a constant concentration of 1% 1-propanol in a fed-batch feeding regime. This addition of the 3HV-related compound 1-propanol resulted in production of a PHBHV copolyester with a molar 3HV fraction of 8 mol.-% (Obruca et al. 2010c).

Apart from oxidative and alcohol-induced stress, also introduction of controlled osmotic pressure can be used as effective tool to boost PHA accumulation in *C. necator*, as it was reported by Pasanha et al. (2014). According to their study, NaCl concentration in cultivation media precisely controlled at 9 g/L enhanced PHA productivity by about 30%. Nevertheless, when NaCl concentration reached 12 or even 15 g/L, PHA accumulation in bacterial cells was considerably inhibited. The positive effect of osmotic pressure on PHA accumulation seems to constitute a general feature applicable on numerous PHA producers since improvement of PHA production by mild osmotic up-shock was also observed for other bacterial strains such as *Pseudomonas fluorescens* (Khare et al. 2014), *Zobellella denitrificans* (Ibrahim and Steinbuchel, 2009), the diazotroph bacterium *Rhizobium* DDSS-69 (Kamnev et al. 2005), or the cyanobacterium *Spirulina subsalsa* (Shrivastav et al., 2010).

Only recently, it was demonstrated that PHB's monomer, 3HB, serves as compatible solute and, moreover, displays a protective effect to lipase. In the presence of 3HB, lipase is sheltered not only against denaturation caused by heat, but also against the oxidative impairment occurring by exposure to H_2O_2 or metal ions like Cu^{2+}. This chaperon-like influence was more effective than that of the well-known chaperone trehalose, and in the same range than described for the chaperone activity of hydroxyectoine. If taking into account that the PHB-positive strain *C. necator* H16 accumulates 16.5-fold higher intracellular 3HB concentration than the PHB-negative mutant strain *C. necator* PHB−4, it is self-evident that the cyclic nature of PHB synthesis and breakdown serves for a sufficient intracellular 3HB pool, thus improving the stress resistance of PHA-accumulating bacteria. The utilization of 3HB as an effective stabilizer in enzyme formulations was proposed by the authors as a possible industrial application of this PHA building block (Obruca et al. 2016b).

By the way, the extraordinary tolerance of *C. necator*'s tolerance to high Cu^{2+} concentrations was the origin of this metalophilic bacterium's name: Translated from Latin, the terminus "*Cupriavidus necator*" describes a "*killer persistent against high copper concentrations*" (Vandamme and Coenye 2004). The high PHA-accumulation potential, combined with its high metal resistance gave rise to intensified search for other metalophile PHA production strains. Chien et al. reported further indication that microbial tolerance to elevated heavy metal levels might be directly connected to PHA accumulation. A strain with high phylogenetic similarity to *C. necator*, namely *Cupriavidus taiwanensis* EJ02, was cultured by these authors in media containing Cd^{2+} ions. When cultivated in complex media, the strain *C. taiwanensis* EJ02 endured Cd^{2+} ions to concentrations up to 5 mM, which is about the five-fold concentration tolerated by PHA-negative mutants of *C. taiwanensis*. Cd^{2+} tolerance of *C. taiwanensis* EJ02 was further improved to concentrations up to 7 mM when supplying defined carbon sources, which at the same time enhances PHB biosynthesis by this strain (Chien et al. 2014). The fact that exposition of bacterial cells to heavy metals induces PHA accumulation was also detected by Pal and Paul (2012), who isolated two bacterial strains identified as *Cupriavidus pauculus* KPS 201 and *Bacillus firmus* AND 408. In both isolates, introduction of high concentration of Ni^{2+} stimulated PHA biosynthesis. Similarly, heavy metals such as Co^{2+}, Cu^{2+} or Zn^{2+} enhanced PHA accumulation in *Azospirillum brasilense* (Kamnev et al., 2005). These findings are analogous to studies with various microalgae, which were shown to efficiently thrive in milieus heavily contaminated by heavy metals such as Cd^{2+}, Pb^{2+}, Cr^{6+}, or other toxic compounds like, e.g., formaldehyde. Similar to boosted PHA accumulation shown for *C. taiwanensis*, such microalgae produce several intriguing products such as pigments or valued lipids under described stress conditions (reviews by Koller et al. 2012, 2014). Nevertheless, it is unlikely that application of heavy metals as stress factors to enhance PHA production in industrial production of PHA is viable since contamination of wastewaters coming out of biotechnological

process could represent serious environmental problem which can be hardly compensate by slight improvement of process productivity.

CONCLUSION

The chapter reveals that a steadily growing number of phylogenetically highly versatile extremophilic strains hold promise for new concepts of efficient PHA production on industrially relevant scale. Up to date, only a limited number of these new concepts has been tested under reproducible, controlled cultivation conditions in laboratory bioreactors operated at different scale and by using different operational regimes, such as discontinuous batch- and fed-batch bioreactor processes, advanced cultivation techniques like cyclic batch and cyclic fed-batch processes, or continuous chemostat cultivations. PHA biopolyesters isolated from the biomass of extremophiles often exhibit material properties at least competitive with the polymer characteristics described for PHA from mesophilic production strains. From an economic point of view, it can be assumed that especially such described extremophile production processes hold promise for a soon industrial implementation, which resort to robust, extremophile organism able to utilize abundantly available raw materials as carbon source, and which can be operated on continuous mode for extended periods. Taking into account that a number of easily accessible, abundantly available, and inexpensive feedstocks are available to be used for processes of "White Biotechnology", e.g., for PHA-production, the next steps in process optimization should be devoted to achieve higher energy efficiency in the large scale bioprocesses; the application of extremophiles appears especially appealing in this context due to the possibility to run such processes under restricted efforts for heating, cooling, or sterility precautions.

However, there are still obstacles to be solved on the way towards the implementation of extremophilic PHA production strains on a routine basis. Further microbial screening studies are needed in order to discover new extremophile production strains, which display growth and PHA accumulation kinetics similar or even superior to the kinetics of mesophilic strains, and, in parallel, are capable to utilize cheap raw materials without the need for excessive upstream processing; in best case, such waste streams should be used, which currently have to be disposed of in a cost-demanding fashion. Additionally to extremophile wild type organisms found in diverse unfriendly habitats, the application of genetic engineering might in some cases be reasonable, to optimize production strains, e.g., by closing catabolic bottle necks which hamper the conversion of selected raw materials by the wild type organism, by knocking out depolymerase-encoding genes, by amplifying the number of PHA synthase genes, or by inserting nuclease genes to decrease viscosity of cell debris-PHA granules suspension

Table 1. Overview on most promising PHA production processes by extremophiles

Microorganism (Type of microbe)	Extreme cultivation conditions; carbon source	Type of PHA produced	Production scale and PHA Productivity	Reference
Haloferax mediterranei (Haloarchaeon)	Halophile: 25% marine salts Starch (20g/L) Glucose (10 g/L)	Copolyesters: PHBHV (in original literature: "PHB")	Stable (monoseptic) continuous cultivation over 3 months in 1.5 L bioreactor; T = 38°C 6.5 g/L PHA on starch 3.5 g/L on glucose	Lillo and Rodriguez-Valera 1990
Hfx. medierranei (Haloarchaeon)	Halophile: 15% NaCl; Glucose plus yeast extract	Copolyester: PHBHV	10 L bioreactor; fedbatch feeding strategy; 0.21 g/(L·h)	Koller et al. 2015b
Hfx. mediterranei (Haloarchaeon)	Halophile: 20% NaCl; Hydrolyzed whey permeate Hydrolyzed whey permeate plus GBL	Copolyesters: PHBHV P(3HB-*co*-3HV-*co*-4HB)	42 L bioreactor; 0.09 g/(L·h), 12.2 g/L PHBHV) 0.14 g/(L·h), 14.7 g/L (3HB-co-3HV-co-4HB)	Koller et al. 2007b
Hfx. mediterranei (Haloarchaeon)	Halophile: 20% NaCl; Hydrolyzed whey permeate, spent fermentation broth of previous whey-based processes	Copolyester: PHBHV	10 L bioreactor batch process 0.04 g/(L·h), 2.28 g/L PHA	Koller 2015
Hfx. mediterranei (Haloarchaeon)	Halophile: 15%; Crude glycerol phase; Crude glycerol phase plus GBL	Copolyesters: PHBHV P(3HB-*co*-3HV-*co*-4HB)	42 L/10 L bioreactor; 0.12 g/(L·h), 16.2 g/L PHA 0.10 g/(L·h), 11.1 g/L PHA	Hermann-Krauss et al. 2013
Hfx. mediterranei (Haloarchaeon)	Halophile: 23.4% NaCl; Extruded rice bran plus extruded corn starch	Copolyesters: PHBHV	5 L bioreactor; pH-stat feeding strategy; 24.2 g/L PHA	Huang et al. 2006
Hfx. mediterranei (Haloarchaeon)	Halophile: 23.4% NaCl; Extruded corn starch plus yeast extract	Copolyesters: PHBHV	5 L bioreactor; pH-stat feeding strategy;	Chen et al. 2006
Natrinema pallidum JCM 8980 (Haloarchaeon)	Halophile: 25% NaCl; Starch	Copolyesters: PHBHV	Shaking flak cultivations; PHA content in CDM ~ 53.14 wt.-%	Danis et al. 2015
Halogeometricum borinquense E3 (Haloarchaeon)	Halophile: 20% NaCl; Sugarcane bagasse hydrolysates	Copolyesters: PHBHV	Shaking flak cultivations; PHA content in CDM of between 45 and 50 wt.-%	Salgaonkar and Bragança 2017

Table 1. (Continued)

Microorganism (Type of microbe)	Extreme cultivation conditions; carbon source	Type of PHA produced	Production scale and PHA Productivity	Reference
Halogranum amylolyticum (Haloarchaeon)	Halophile: 20% NaCl; Glucose	Copolyesters: PHBHV (20 mol.-% 3HV!)	7.5 L bioreactor; fed-batch feeding strategy; 0.074 g/(L·h), 14 g/L PHBHV	Zhao et al. 2015
Halomonas campaniensis LS21 (Gram-negative bacterium)	Halophile and alkaliphil: 27 g/L NaCl and pH-value 10; Alkaline seawater and artificial carbonaceous kitchen waste	Homopolyester: PHB	Open cultivation for 65 days 26% PHA in CDM (wild type strain), 70% PHA in CDM (engineered strain)	Yue et al. 2012
Halomonas TD01 (Gram-negative bacterium; wild-type)	Halophile and alkaliphile: 60 g/L NaCl and pH-value 9; Glucose	Homopolyester: PHB	Open continuous cultivation process for 56 h; 64 g/L PHA	Tan et al. 2011
Halomonas TD01 (genetically engineered) (Gram-negative bacterium)	Halophile: 60 g/L NaCl; Glucose, propionic acid	PHB, PHBHV	Open continuous cultivation process for 56 h; 80 g/L PHB, 56 g/L PHBHV	Tan et al. 2014
Halomonas venusta KT832796 (Gram-negative bacterium)	Moderate halophile: 15 g/L NaCl (moderately halophile); Glucose	Homopolyester: PHB	2 L bioreactor; fed-batch feeding strategy (pH-stat or single pulse feeding, respectively)	Stanley et al. 2017
Bacillus megaterium uyuni S29 (Gram-positive bacterium)	Halophile: 45 g/L NaCl; Glucose	Homopolyester: PHB	Shaking flak cultivations; 2.2 g/L PHB, 0.10 g/(L·h)	Rodriguez-Contreras et al. 2013c
Pseudomonas extremaustralis (Gram-negative bacterium)	Psychrophile: 28°C optimum, but growth also at lower temperature; Contaminated soil samples	Homopolyester: PHB	Static cultivation on poly(styrene) biofilms biofilm vs. shaking flask cultures PHA productivity: n. r.	Tribelli et al. 2011, Tribelli et al. 2012

Microorganism (Type of microbe)	Extreme cultivation conditions; carbon source	Type of PHA produced	Production scale and PHA Productivity	Reference
Thermus thermophilus (Gram-negative bacterium)	Thermophile: 75°C; Gluconate Octanoate	Copolyesters: *mcl-co-scl*-PHA *scl-co-mcl*-PHA	Shaking flask cultivations; Up to 40% PHA in CDM	Pantazaki et al. 2003
Synechococcus sp. MA19 (Gram-negative cyanobacterium)	Thermophile: 50°C; 2% CO_2	Homopolyester: PHB	Photoautotrophic cultures farmed in simple bottles; 20- 27% PHA in CDM	Miyake et al. 1996
Spirulina sp. MA19 (Gram-negative cyanobacterium)	Thermophile: 50°C; CO_2	Homopolyester: PHB	Autotrophic cultivation 2.4 g/L PHB	Nishioka et al. 2001
Chelatococcus sp. strain MW10 (Gram-negative bacterium)	Thermophile: 55°C; Glucose	Homopolyester: PHB	2 Lbioreactor; fedbatch fermentation (ca. 3 g/L PHB); 42 L bioreactor; cyclic batch fermentation(ca. 4 g/L PHB); 42 L bioreactor; cyclic fed-batch fermentation (ca. 16.8 g/L PHB)	Ibrahim and Steinbüchel 2010
Chelatococcus daeguensis TAD1 (Gram-negative bacterium)	Thermophile: 50°C; Glucose Glycerol	Homopolyester: PHB	Shaking flask cultivations 3.44 g/L PHB 80% PHB in CDM	Xu et al. 2014
Chelatococcus daeguensis TAD1 (Gram-negative bacterium)	Thermophile: 50°C; Glycerol plus cocktail of nitrogen sources	Homopolyester: PHB	Two-stage fedbatch process; 17.4 g/L PHB, 0.434 g/(L·h)	Cui et al. 2015
Bacillus shackletonii K5 (Gram-positive bacterium)	Thermophile: 45°C; Glucose	Homopolyester: PHB	Cultivation in batch mode 7 g/L PHB	Liu et al. 2014

Abbreviations: CDM: Cell dry mass; GBL: γ-Butyrolactone; *Hfx.*: *Haloferax*; *mcl*-PHA: medium chain length PHA; PHA: Polyhydroxyalkanoate; PHB: Poly(3-hydroxybutyrate); PHBHV: Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate); *scl*-PHA: short chain length PHA.

after cell disruption, thus facilitating downstream processing. Such strain improvement by genetic engineering of halophiles is reviewed in this chapter for the case of *Halomonas* sp. Moreover, deleting genes encoding enzymes responsible for side-product formation contributes to achieve higher carbon-to-product conversion yields and thus high atom-economy, as successfully shown for EPS-negative mutants of the haloarchaeal PHA production strain *Hfx. mediterranei*. In addition, the deletion of depolymerase-encoding genes paves the way to further increase productivity by inhibiting intracellular biopolyester degradation especially during the later process phase, as it was demonstrated in the case of PHA biosynthesis by the thermophile organism *Chelatococcus* sp., or for the halophile strain *Halomonas* sp.

Beside all the positive features of extremophiles, we need to be aware of the fact that extreme cultivation conditions typically call for special bioreactor equipment and adaptation of the bioreactor's auxiliaries. This encompasses better-quality reactor materials, enhanced sealing systems, and highly resistant sensors and probes. Hence, additional engineering efforts and material testing and improvement will be needed.

In spite of all these challenges we are still confronted with, lab-scale experimental results and ongoing pilot scale R&D activities make it likely that the implementation of extremophile organisms for biosynthesis of PHA and other vendible products belongs to the "hot topics" of "White Biotechnology" in the very next future. Table 1 gives an overview on selected case studies presented in this chapter, summarizing the production strains, the nature of extreme conditions and type of carbon sources used, the monomeric composition of the produced PHA, production scale, and biopolyester productivity.

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Chapter 2

POLYHYDROXYALKANOATES: NEW BROWSING THE PHA BIOSYNTHESIS INSIGHTS IN NATIVE AND RECOMBINANT STRAINS

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ABSTRACT

Polyhydroxyalkanoates (PHAs), a biodegradable and biocompatible class of biopolymers, gained an increased interest nowadays as potential alternatives to synthetic polymers. They are synthesized as intracellular carbon and energy storage compounds from over 300 species, mainly bacteria, in the presence of excess carbon and under oxygen, nitrogen or phosphorus limitation, or after pH shifts. Although most bacteria accumulate PHAs under stress conditions, there are some of them that do not require nutrient limitation for PHA synthesis such as *Alcaligenes latus*, or recombinant *E. coli.* In the first case, PHAs are degraded and used for bacterial growth when a limiting nutrient is provided. Therefore, bacteria that produce PHAs have both biosynthetic and degrading enzymes. For PHAs biosynthesis three pathways have been elucidated so far. In pathway I, 3HB monomers are generated by the condensation of two acetyl-CoA molecules from the tricarboxylic acid (TCA) cycle. In the second pathway (II) the substrates are generated by β-oxidation of fatty acids, while in pathway III monomers are generated from structurally unrelated and simple carbon sources such as glucose, sucrose and

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fructose. Gene's organization of the corresponding biosynthetic enzymes also differs in PHAs producing bacteria. The key enzyme is PHA synthase and significant effort has been made to generate synthases with enhanced activity and substrate specificity. More than 150 monomers have been reported so far; their composition influences the physical properties and characteristics of the polymer, thus particular notice is given to the carbon source that will be used. Recombinant strains also provide an alternative way to produce PHAs with novel properties from inexpensive raw materials. The plethora of enzymes, regulators as well as their genes involved in PHAs biosynthesis will be discussed in details.

Keywords: polyhydroxyalkanoates, PHA biosynthesis, PHAome, PHA granules, PHA gene organization, phasins

1. INTRODUCTION

Polyhydroxyalkanoates (PHA) have grown a lot of interest due to their many applications such as biomaterials and bioplastics. PHAs are considered a polyester group synthesized by microorganisms, particularly by more than 90 genera and 300 bacteria species that can be divided into two groups. The first one requires stress conditions to produce PHAs such as an essential nutrient limitation or a pH shift parallel to carbon excess, with the main representative *Ralstonia eutropha*. Bacteria of the second group produce PHAs naturally without any nutrient constraint and the main representatives are *Alcaligenes latus* and recombinant *Escherichia coli* [1, 2]. Large quantities of PHAs are synthesized by wild-type strains as well as by recombinant strains, while various substrates counting sugars, fats and oils, industrial wastes, wastewater, and glycerol have been used for PHA production [3].

The biosynthesis of PHAs is favored compared to their chemical production~~, as~~ since in the first case polymers with higher molecular weight are formed. However, PHA synthase, the key enzyme in PHA biosynthesis, exhibits substrate specificity and much less control of the produced monomers is allowed [4, 5]. The biosynthesis is mainly conducted by microorganisms in solutions containing a sustainable carbon source (starch, fatty acids, etc.) leading to the production of more than 150 different PHA monomers arranged into homopolymers, random copolymers and block polymers depending on the growth conditions [5]. PHA depolymerization is also applied for the production of PHA monomers such as (R)-3-hydroxyalkanoic acids when convenient environments are provided, for instance, pH 3-4 [6], or pH 11 [7], or knockout mutations of the PHA synthesis operon occur [5, 8].

PHA biosynthesis is not a simple bacterial cellular process of a family of biomolecules that is not yet fully elucidated; however, new information emerges and enriches this knowledge. It is the orchestrated effect of the action of many bacterial components (entire metabolic pathways, genes, proteins, enzymes, regulators etc.), that

have to act at a particular time of a particular cell state. A new analogous term with these of genome, transcriptome, and proteome defining the whole clusters of genes, all nucleic acids, and transcripts, and the total of proteins respectively, existent into a cell or in a population of cells at a specified time, it appeared – the 'PHAome.' The 'PHAome' reflects the variety of monomers, homopolymers, random and block copolymers, functional and graft polymers, molecular weights, and their combinations, as well as also the spectrum of PHAs with diverse molecular weights and monomer proportions that subsist at a particular time moment in a bacterial cell [9].

This review hopes to touch the major of these components involved in this cellular bacterial process of PHA biosynthesis, focusing mainly on modern literature as it is impossible to cover all the breadth of information in this field. The 'PHAome' comprehension will contribute to the uncovering of new PHAs properties and applications providing advanced materials of this family.

2. PHA BIOSYNTHESIS PATHWAYS

Many enzymes are involved either directly or indirectly in PHA biosynthesis. Three biosynthetic pathways have been well-established so far (Figure 1). In the first pathway (I) β-ketothiolase (*phaA*) condensates two molecules of acetyl-CoA, originated from the TCA cycle, into acetoacetyl-CoA. An NADPH-acetoacetyl-CoA reductase (*phaB*) converts acetoacetyl-CoA into 3-hydroxybutyryl-CoA and finally PHA synthase (*phaC*) forms ester bonds and polymerizes the monomers into P(3HB). A related PHA degrading pathway consists of a PHA depolymerase, a hydrolase, a dehydrogenase and an acetoacetyl-CoA synthase [5, 10].

Pathway II is used by some species of *Pseudomonads* for medium-chain length (mcl) PHAs, or PHA copolymer synthesis of (R)-3-hydroxybutyrate and (R)-3-hydroxyhexanoate. In this pathway, the substrates originate from the β-oxidation of fatty acids. A 3-ketoacyl-CoA reductase, an epimerase, an (R)-enoyl-CoA hydratase, an acyl-CoA oxidase, and an enoyl-CoA reductase are required for 3-hydroxy acyl-CoA synthesis. The resultant monomers are then polymerized by PHA synthase. In the third pathway (III) simple carbon sources are exploited to produce (R)-hydroxy acyl-CoA monomers with the aid of acyl-ACP-CoA transacylase [3]. In pathway III, (R)-3-hydroxy acyl-ACP is the main precursor molecule of 3-hydroxy acyl-CoA that leads to PHA synthesis [5, 10–12] (Figure 1).

Some microorganisms use different pathways to produce PHAs. For example, *Aeromonas caviae* lacks a thiolase and a reductase employing an enoyl-CoA hydratase to synthesize (R)-3-hydroxy monomers from fatty acids oxidation. This microorganism is able to form various copolymers depending on the fatty acids that are used (odd or ever carbon number) [13]. In *Nocardia corallina* the PHA monomers are produced from fatty

acid catabolism and from the three-step PHB biosynthesis pathway. The 3HV monomers derive from methyl malonyl-CoA pathway initiating from succinyl-CoA [14, 15].

In summary, five more pathways can lead to PHA biosynthesis except for the three basic pathways described above. In pathway IV, an enzyme NADH-dependent an acetoacetyl-CoA reductase participates by oxidizing the precursor molecule for the PHA generation, the substrate (S)-3-hydroxybutyryl-CoA. In pathway V, that was mentioned in *Clostridium kluyveri* [5, 16] the enzyme 4-hydroxybutyrate-CoA: CoA transferase catalyzes the transformation of the substrate succinyl-CoA derived from the TCA cycle, to produce 4-hydroxybutyryl-CoA. In pathway VI, a variety of substrates 4,5-hydroxy acyl-CoA are employed for PHA synthesis [5, 17]. In pathway VII, the metabolite 1,4-butanediol is oxidized to produce 4-hydroxybutyrate, which consequently is transformed to the PHA precursor compound 4-hydroxybutyryl-CoA and then also to the PHA precursor substrate 4-hydroxyhexanoyl-CoA [5]. Finally, in pathway VIII eight enzymes are involved and use cyclohexanol that it is turned into a 6-hydroxyhexanoate PHA [5].

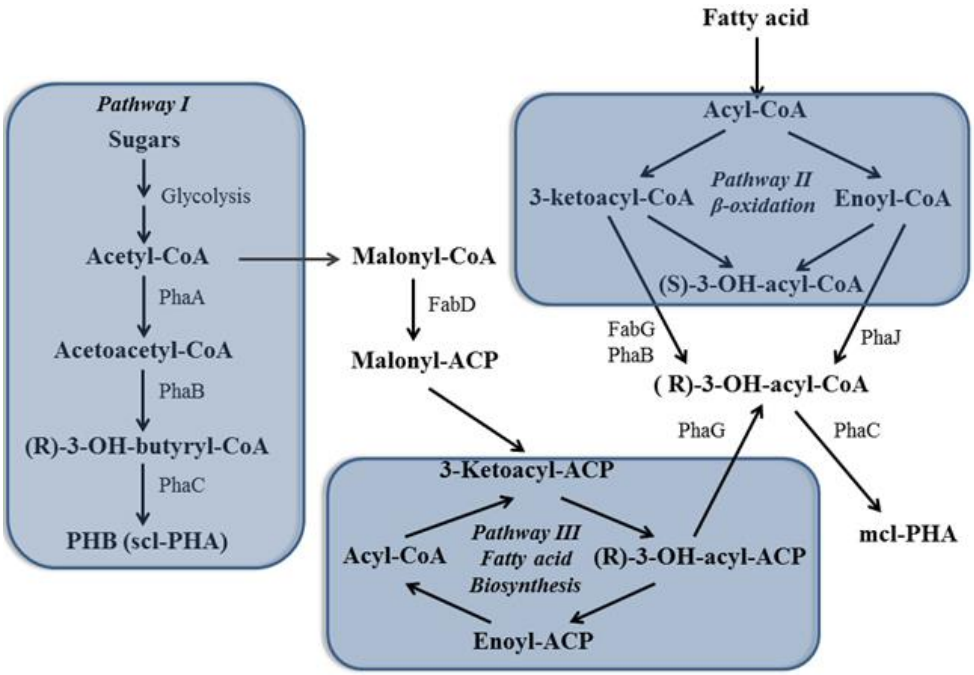


Figure 1. The three basic PHAs biosynthetic pathways.

Depending on the fatty acids composition, bacteria produce PHA monomers consisting either by the same length of carbon atoms with the carbon source, or by 2, 4, or 6 carbons shortened, or they produce a mixture of PHA monomers that is a reflection of the carbon source used [18, 19]. The dependence of the PHA monomers composition from the fatty acids used indicates a direct relevance with the fatty acid oxidation pathway. The mcl-PHA polymerase uses as a substrate the 3-hydroxy acyl-CoA which

can be originated from the β-oxidation pathway intermediates. This conversion requires a hydratase, an epimerase, or a reductase, depending on the nature of the intermediates [19]. Studies from *Pseudomonas oleovorans* and *Pseudomonas aeruginosa* showed the existence of two mcl-PHA polymerases with 50% amino acid identity as well as an identical activity for PHA synthesis from fatty acids [15, 20, 21].

Experiments conducted with *P. putida* grown in different temperatures and provided with glucose as the sole carbon source revealed a change in the ratio of unsaturated and saturated monomers of membrane lipids and PHA products. This finding suggests that PHA production from glucose is related to fatty acid biosynthesis [22]. In this process, a conversion of (R)-3-hydroxy acyl-ACP to (R)-3-hydroxy acyl-CoA is required by an enzyme encoded by *phaG* gene [15, 23].

An attempt to improve mcl-PHA production with specific monomer composition was performed in the study of Gao and collaborators [24]. They managed to increase the 3-hydroxydecanoate content in the synthesized mcl-PHAs from *P. putida* KT2440. To achieve this, β-oxidation was suppressed either by a *fadBA* knockout mutant or by modifying the carbon and energy sources of the wild-type strains. In the second case, a 74% of PHA accumulation per dry cell weight and a 97 mol% of 3-hydroxydecanoate was succeeded when acrylic acid was included in the culture media. This suggested that the monomer load can be altered by acrylic acid adjustment. In addition, high yield of PHA production occurred when decanoic acid was supplemented compared to alteration of acetate for a *de novo* synthesis of 3-hydroxydecanoate monomers [24].

3. PHA BIOSYNTHETIC ENZYMES

3.1. PHA Synthase/Polymerase

Generally, PHAs synthases, the most crucially-operating PHAs biosynthetic enzymes, use as substrates various HA (hydroxyl alkanoates)-CoAs and incorporate the suitable monomer units, undergoing polymerization, to the PHAs chain. PHA synthase or polymerase can be found both in hydrophilic, which is less active and in hydrophobic (granules) state. P(3HB) polymerase forms ester bonds between 3HB monomers. PHA polymerases resemble lipases since they catalyze formation and hydrolysis, respectively, of ester bonds at the hydrophobic and hydrophilic interface. In addition, an active cysteine site is located in the lipase box (Gly-X-Cys-S-Gly-Gly) they possess. *In vitro* and *in vivo* studies revealed that when the amount of PHA synthase is decreased the molecular weight of the produced polymer is increased, therefore they are able to control the molecular weight of the synthesized P(3HB) [15, 25].

They are categorized into four classes (class I, II, III, and IV) according to their subunit composition and substrates specificities [26]. Class I PHA synthases, like those

derived from *R. eutropha* and *A. latus*, prefer short chain length (scl) HA-CoAs (C3-C5) for incorporation in the polymeric chain [27, 28]. Class II PHA synthases prefer medium chain length (mcl) HA-CoAs (C6-C14) as monomer unit for the polymerization and the main representatives are *Pseudomonads* species [20]. Some others class II PHA synthases derived from *Pseudomonas* sp. 61–3 [29] and *Pseudomonas* sp. 6–19 [30] incorporate both kind of scl- and mcl- monomers, but with a very small preference towards scl-monomers. Class III PHA synthases have substrate specificity towards scl HA-CoAs, like class I synthases, and are constituted from two different subunits PhaC and PhaE [31]. They are extremely specific to recognize as substrates scl HA-CoAs, but incorporate also mcl HA-CoAs in cases that are expressed in some *Pseudomonads* [32]. Finally, class IV PHA synthases consist of PhaC and PhaR subunits, which are usually appeared in *Bacillus* strains synthesizing P(3HB) [33–35]. Among the PHA synthases of various microorganisms that have been studied, 15 residues are conserved. A possible explanation for the high diversity of PHA synthases is the wide variety of stress conditions that lead to PHA formation, requiring enzymes with different structural properties. A multiple alignment of the primary sequence of 59 PHA synthases was performed only in regions containing conserved amino acid residues and revealed that eight strictly conserved residues possess an overall identity of 8-96% [36]. A conserved tryptophan residue is involved in protein–protein interactions, while in the majority of class II synthases a histidine residue, located after a catalytic aspartate, and is also involved in the catalysis [36].

3.1.1. Characteristics of PHA Synthase: Size, Structure and Subunit Composition

PHA synthase is active when aggregates of more than one subunit are formed. The formation of protein complexes of 10-11 nm diameter was reported in *Chromatium vinosum*, and they are located at the surface of PHA granules [37]. Electron microscopic data revealed that the holoenzyme of native PHA synthase exhibited a high molecular weight of about 390 and 400+20 kDa forming particles of 11.2-12.8 nm in diameter [38], or 360+50 kDa, or 520+50 kDa [39]. Therefore, the active holoenzyme of *C. vinosum* PHA synthase consists of 4 PhaCCv and 6 PhaECv subunits [38].

3.1.2. Substrate Specificity of PHA Synthase

PHA synthases have a very broad spectrum of substrates that can be used, which justifies the huge spectrum of hetero-polymers PHAs. Generally, PHA synthase specificity is considered low in respect of the position of the hydroxyl group, the existence of substitutions, the position of double bonds and the length of the hydroxyl-alkyl moiety of the CoA thioesters. In contrast, the stereospecificity of PHA synthases is very high since the hydroxyl carbon atom should be in the R-configuration [40]. Nevertheless, concerning the length of the hydroxyl-alkyl moiety of the CoA thioesters

that incorporates in the polymer chain usually operate with scl HAs, or mcl HAs. Representative PHA synthases and their characteristics in respect of subunits number, molecular weight, substrates preference, and species are shown in Table 1.

3.1.3. PHA Synthase Crystallization

Until now a lack of available information concerning three-dimensional structural of PHA synthase PhaC limited the full understanding of its catalytic function in PHA polymerization process. The first case of PHA synthase, which has been crystallized at 1.8 Å resolution is that from *R. eutropha* (RePhaC1), and the mechanism for PHA synthesis based on its structure has been very recently reported. The enzyme is in an active dimeric form, while the C-terminal domain of the enzyme is responsible for the polymerization catalysis. The catalytic triad of Cys-His-Asp amino acids participates in the catalysis via a non-processive ping-pong mechanism. Comparative analysis elucidates that the structural features-based differences might be responsible for the difference of substrate specificities among the various PHA polymerases. Moreover, a RePhaC1 mutant with increased PHA synthase activity has been constructed based on the structure - function relationship [42]. These data suggest the potentiality to develop novel strategies in the production of tailor-made PHAs possessing diverse monomers composition and desired material properties.

The crystal structure of the catalytic domain of PhaC from *Chromobacterium* sp. USM2, PhaCCs-CAT at high-resolution appeared recently. The enzyme forms a fold of α/β hydrolase comprising α/β core and CAP subdomains. The catalytic triad Cys291, Asp447, and His477, is located at the bottom of a cavity, full of water molecules and covered by the partly disordered CAP subdomain. This structure was designated as the closed form, and it differs from the recently reported catalytic domain of *R. eutropha* (PhaCCn-CAT). The structural comparison showed that the last taking a partially open form might preserve a narrow tunnel for substrate access to the active site, but no product withdrawal. PhaCCs-CAT forms a “face to face” dimeric form intervened by the CAP subdomains and distinct from this of PhaCCn-CAT. It is suggested that CAP subdomain subsist a conformational change during the catalytic stage that implicates rearrangement of the dimer to accommodate substrate entrance to the active site, product formation and then its release [43].

3.1.4. Catalytic Mechanism of PHA Synthase

The catalytic mechanism of PHA polymerization and the PHA chain elongation was previously extensively reviewed [36] and references therein). Recently it was reported that the active site of *R. eutropha* PHA synthase is located at the monomeric form of the C-terminal domain of PhaC1 (RePhaC1CD). It is constituted from a well-conserved catalytic triad of amino acids Cys-His-Asp, which operates as a covalent nucleophile, a general base, and an electron donor, respectively. Their contribution to catalysis was

proven by mutating these amino acids to alanine leading in the total loss of PhaC1 activity both by *in vitro* and *in vivo* experiments. The recently proposed catalytic mechanism of P(3HB) polymerization reaction by PHA synthase occurs in two stages: firstly the covalent binding of 3-HB to the active site and later on P(3HB) chain elongation, as described in details by Kim et al. [42].

Moreover, the 3D reconstructed model of the full-length PhaC1 of *R. eutropha* by small angle X-ray scattering (SAXS) analysis was reported [42]. PhaC1 contains a dimer catalytic C-terminal domain (CD) of RePhaC1 located in the center of the full-length enzyme and the N-terminal domain (ND) in the opposite side of the dimerization subdomain designating that it is not implicated directly in the enzymatic catalysis. However, its role in PHA polymerization process is crucial since the ND directs the enzyme to the PHA granules and stabilizes the polymer chain near the CD, where is the active site. According to successive truncation studies on the ND, it was indicated that five of the predicted α-helices (N-α3 to N-α7) are necessary for the appropriate folding and the granule binding of this domain [44]. Moreover, PhaM protein forms a complex with PhaC1 through interaction with its ND and thus activates the enzyme by offering a more spacious surface area for interaction with the polymer chain. The catalytic mechanism of PHA polymerization reaction by RePhaC1 is presenting in details in Figure 2.

3.1.5. The Lag Phase of Polymerization Reaction by PhaC

A lag phase in the polymerization reaction was discovered by kinetic studies of *R. eutropha* PHA synthase (RePhaC) catalysis [31, 45, 46]. It was found that the lag phase is dependent on the enzyme concentration and it can be diminished, whereas the enzyme activity increased by artificially priming of the enzyme with a trimer or saturated trimer of HBCoA ((HB)3CoA), or an analogue of $(HB)_3$-CoA HB), where the terminal OH group is replaced with H (sTCoA) [31, 47]. It is also suggested that priming of the synthase shifts the equilibrium of the monomeric – dimeric form to the dimeric, which is the active one [47].

As recently described by Kim et al. [44] the N-terminal domain of *R. eutropha* PHA synthase (RePhaC1ND) might justify the reason for the lag phase reduction upon the addition of PHB polymer. The developing P(3HB)n polymer may interact with RePhaC1ND and supplementary forces of the enzyme and the second substrate are developed. Binding of the P(3HB) polymeric chain to RePhaC1ND leads to its increased accessibility to the RePhaC1CD active site and enhancement of the enzyme activity is observed. Thus, the reduction of the lag phase after the provision of $(3HB)_3$ might be attributed to the reduced time required to attain a certain chain length permitting its binding to RePhaC1ND [44].

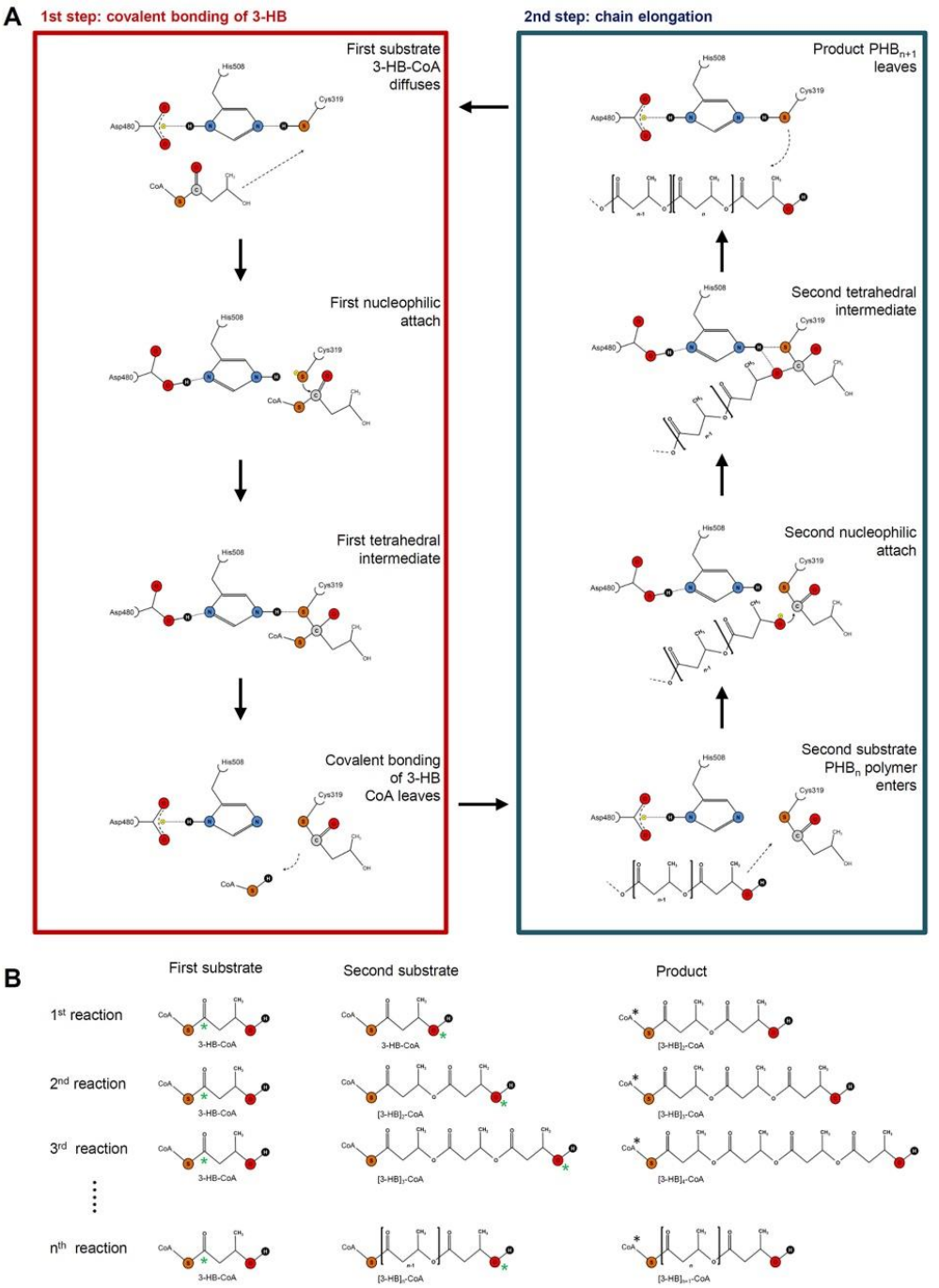
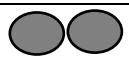
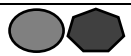
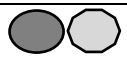
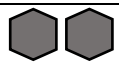


Figure 2. Catalytic mechanism of PHA polymerization reaction by RePhaC1. (A) Catalytic mechanism of RePhaC1. The polymerization reaction occurs into two steps, a covalent bonding of 3-HB and PHB chain elongation. (B) Initial PHB polymerization. The first and second substrates and production during the initial PHB polymerization phase are shown. Permission from ref. [42].

Table 1. Representative PHA synthases and their characteristics in respect of subunits number, Molecular Weight (MW), substrate preference, species

Class	Subunits	MW	Substrate preference	Species	References
I	 PhaC PhaC	PhaC (64 kDa)	scl-HA-CoAs	*Ralstonia eutropha*, *Alcaligenes latus*	[27]
II	 PhaC PhaC	PhaC (63 kDa)	mcl-HA-CoAs mcl-HA-CoAs > scl-HA-CoAs	*Pseudomonas* species *Pseudomonas* sp. 61-3, *Pseudomonas* sp. 3-19	[20, 29, 30]
III	 PhaC PhaE	PhaC (40 kDa) PhaE (Mr 41)	scl-HA-CoAs	*Allochromatium vinosum* *Thiocapsa pfennigii*	[32, 38]
IV	 PhaC PhaR	PhaC 40-kDa PhaR (22-kDa)	scl-HA-CoAs	*Bacillus* strains	[33, 34]
Other	 PhaXPhaX	PhaX (55 kDa)	lcl-HA-CoAs> mcl-HA-CoAs	*Thermus thermophilus* HB8	[41]

Experimental evidence from size exclusion chromatography, SDS-PAGE, and gel permeation chromatography revealed that PhaC was co-purified unexpectedly with the phasin protein, PhaP1. When PhaC was in monomeric/dimeric (M/D) forms it was not associated with PhaP1, or P(3HB). Experiments to monitor P(3HB) generation on the macromolecular complex indicated no lag phase in PhaC catalysis, in contrast to M/D forms of *R. eutropha* PHA synthase (RePhaC) and *R. eutropha* PhaC purified from *E. coli* (EcPhaC). Therefore, PhaC in the high molecular weight complex is in a P(3HB)-primed form. The existence of both PhaC forms, primed and non-primed suggests that the kinetics of elongation rate for P(3HB) generation is faster than the initiation rate *in vivo* [48]. This suggestion is also supported by *in vitro* studies, which pointed out that PhaC is primed by rapid acylation and elongation of its active site cysteine by HB-CoA. The soluble macromolecular complex PhaC/PhaP1/P(3HB) is capable of adding HB units to the PhaC in a kinetically competent mode [48]. To summarize these observations a modified micelle model for granule synthesis in *R. eutropha* was proposed [48]. The lag phase of the polymerization reaction catalyzed by PHA synthase PhaC observed at the initial stage was ascribed to the low affinity of the enzyme PhaC for the nucleotide moiety [45, 48].

3.2. β-ketoacyl-CoA Thiolase

In PHA biosynthesis β-ketoacyl-CoA thiolase catalyze the thiolytic cleavage of 3-oxoacyl-CoA into acyl-CoA (shortened by two carbon atoms plus acetyl-CoA), as shown in Figure 3 [49]. β-ketoacyl-CoA thiolase contains two active cysteines. There are two groups of thiolases: the first group contains enzymes involved in fatty acid degradation and the second is involved in ketone formation, steroid and isoprenoid formation and P(3HB) biosynthesis. They are found in prokaryotes as well as in yeast and higher eukaryotes [15, 50].

The native enzyme of β-ketoacyl-CoA thiolase from *T. thermophilus* is a multimeric protein of 182 kDa, consisting of four identical subunits of 45.5 kDa [49]. A substrate inhibition was observed at high concentrations; when one of the substrates (acetoacetyl CoA or CoA) is varied, while the concentrations of the second substrate (CoA or acetoacetyl CoA respectively) remain constant. The proposed mechanism is a ping-pong mechanism. Experimental evidence also showed that a cysteine is possibly located at/or near the active site of β-ketothiolase [49]. PhaA from *Ralstonia eutropha* (RePhaA) was recently cloned, purified and crystallized and the structure was solved [51].

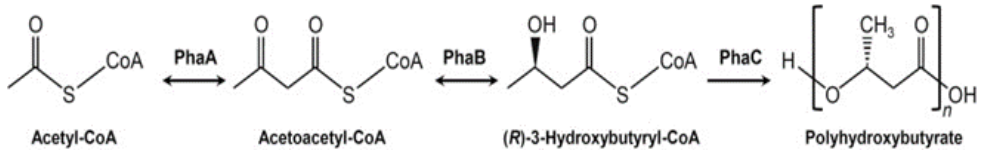


Figure 3. The three step formation of P(3HB) [49].

3.3. Acetoacetyl-CoA Reductase

In *R. eutropha* it is an NADH-dependent dehydrogenase that catalyzes the second step of P(3HB) formation [52]. When the condensation reaction occurs under PHA accumulating conditions thiolase, acts against its thermodynamically favored direction [15, 50].

4. ORGANIZATION OF PHA BIOSYNTHETIC GENES

Many bacteria accumulate PHAs under stress conditions and nutrient imbalance, in granule forms, which can consist of an up to 90% of their total cell dry weight. Depending on the environmental niche bacteria, various different pathways have been developed to optimize PHA production. As mentioned above, the first P(3HB) biosynthetic pathway described, includes three enzymes: i) the β-ketoacyl-CoA thiolase (*phbC*) which is responsible for condensation of two acetyl-CoA into acetoacetyl-CoA; ii) the acetoacetyl-CoA reductase (*phbA*) which is responsible for the reduction to (R)-3-hydroxybutyryl-CoA monomers; and iii) the P(3HB) polymerase (*phbB*) which adds monomers to the already synthesized polymer. In *R. eutropha*, *Pseudomonas acidophila*, *Acinetobacter* spp. the *phbCAB* genes form an operon with the order mentioned not being a necessity [15]. In other bacteria *phbAB* and *phbC* are distinct from one another. In addition, in some bacteria (*Synechocystis*) PHA polymerase is heterodimerized and encoded by two different genes. *Methylobacterium extorquens* [53], *Nocardia coralline* [54], *Rhizobium etli* [55] obtained only the PHA polymerase. In *P. oleovorans* and *P. aeruginosa* two PHA synthesizing genes were found separated by a PHA depolymerase (*phaZ*) [20, 56]. Evolutionary, the PHA biosynthetic pathway was probably a minor biosynthetic pathway and when PHA production became advantageous high-PHA-accumulating strains survived under selective pressure [15].

Bacillus cereus YB-4 demonstrates PHA accumulation activity using distinct PHA biosynthetic gene clustering in comparison to other strains of the same species. There have identified a MaoC-like protein in the *pha* cluster that does not exist in other strains. This protein is an R-specific enoyl-CoA hydratase that is also mentioned as PhaJ when involved in PHA biosynthesis. In this study it was found that PhaJ is responsible for

crotonyl-CoA hydrolysis. In addition, it was found that expression of PhaJ together with PHA synthase in *E. coli* strain resulted in increment PHA accumulation recommending a monomer supplier function of the PhaJ. The monomers included short chain length properties which were preferred as substrates. Moreover, deletion of the *phaJ* gene inhibited the PHA accumulation. The *pha* cluster of *B. cereus* YB-4 exemplifies a new type of *pha* cluster containing the *phaJ* gene [57].

A very common microorganism for mcl-PHA production in industrial range is *Pseudomonas putida* KT2440. In the study of Mozejko-Ciesielska et al. [58] the relationship between stringent response and PHA accumulation was considered. A *relA/spoT* mutant of *P. putida*, unable to induce the stringent response, was studied. It was observed that this mutant was able to acquire mcl-PHAs under optimal and nitrogen restriction case without any sufficient difference. This postulated that stringent response in not a necessity for PHA accumulation when oleic acid is used as the carbon source. The monomer composition was similar using either fatty acids either oleic acid. It was also reported that in *relA/spoT* mutant the *phaI* and *phaF* genes expression was much higher than in the wild type suggesting an implication of the stringent response to the regulation of the latter genes [58].

5. PHB/PHA GRANULES

P(3HB), the most known homo-biopolymer, consists of linear chains of (R)-3-hydroxybutyrate (3HB) units. Three types of P(3HB) were identified, which differ in the number of (R)-3-hydroxybutyrate units and in the functions that execute. i) The high molecular weight P(3HB) constituted from $>10^3$ 3HB units (storage P(3HB)) which are generated and accumulated in the intracellular inclusion bodies or PHB/PHA granules. ii) The low molecular weight P(3HB), which is known as oligo-P(3HB) and consists of 100 to 200 3HB units. iii) The complexed, or conjugated (cP(3HB)) constituted of a low number of 3HB units (<=30) that is covalently linked to proteins [59].

The molecular incidents that take place at the beginning of P(3HB) polymer synthesis and of PHB/PHA granule formation, as well as the complex composition of the PHB/PHA granules surface coating, have only recently become the focus of research and they had not been extremely examined yet. Although, a summary of all the progress in PHA granule formation in *R. eutropha* H16 (PHB producer) and in *Pseudomonas putida* (mcl PHA producer) are presented in Jendrossek and Pfeiffer [59].

Despite the fact that for many years it was believed that the PHAs granules are covered with a phospholipid layer as evidenced by some *in vitro* experiments, its presence was never confirmed by *in vivo* experiments. Recent experiments negate the myth of the presence of phospholipids as a coating layer of the PHA granules and that only the proteins make up the surface layer of the representatives of α-, β- and γ-

proteobacteria. A model for the proteins participating in the *in vivo* PHB granule composition in *R. eutropha* H16 is also shown [60].

5.1. Models of PHB Granules Formation

Concerning PHB/PHA granule formation three models were proposed: i) the Micelle Model, ii) the Budding model and iii) Scaffold model (Figure 4) [59]. The Micelle Model proposes that the cytoplasmic and soluble PHA synthase, in its dimeric active form, operates to generate hydrophobic P(3HB) chain when its substrates 3-hydroxybutyryl-CoA/3-hydroxyalkanoyl-CoA concentration are adequate [61–63]. Due the low solubility and hydrophobicity in the aqueous cytoplasmic environment, the nascent polymeric P(3HB) chains are driven to their aggregation and micelle-resembling structures formation, where the partially hydrophilic/hydrophobic PHA synthase remains on the surface of the polymer [64, 65].

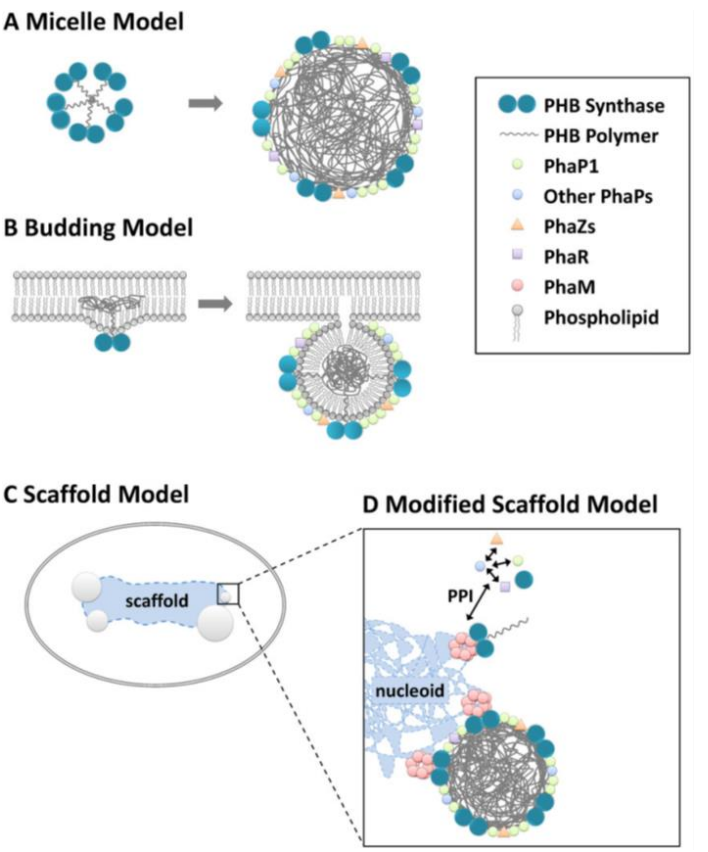


Figure 4. The three models of PHB/PHA granule formation [59].

Subsequently, phasins and others granule-associated proteins (GAPs) are recruited to the developing granules. An after-effect of the micelle model is that the onset of PHB granule generation should have occurred within the cytoplasm in any random localization of the cell. The Budding Model hypothesizes that the PHB/PHA synthase is localized or conjugated directly or indirectly to the cytoplasmic membrane, where the developing polymeric chain is released into membrane bilayer and granules are generated. The Scaffold Model hypothesizes that PHA synthase of growing PHA granules is attached to a yet unidentified scaffold molecule within the cell. This model has replaced the micelle mode of PHB granule formation. The PHB synthase initiation complex is bound to the bacterial nucleoid [59], which is attached to the cell wall and represents the scaffold to which PHA granules are attached.

5.2. Granule-Associated Proteins (GAPs)

PHA granules harbor a significant number of proteins on their polymer surface, nominating that they are not simple storage sacks of carbon and energy but constitute supramolecular complex structures with particular functions and thus, the term ‘carbonosome’ was attributed to them owing to the multi-functionality of PHA granules [66]. Proteome analysis of purified PHB granules from *R. eutropha* H16 revealed a large number of polypeptides (>400) [59]. Numerous of them are enzymes for which their role have not been reported except the most known PHA synthase, and PHA depolymerase. A distinction in 4 classes of the granule-associated proteins (GAPs) that have been identified so far was proposed, namely PHA synthases, PHA depolymerases, phasins and other proteins [67] that includes diverse transcriptional regulators as well as enzymes involved in the synthesis of PHA monomers, hydrolases, reductases and others [59, 68].

Four GAPs were found in *R. eutropha* H16, the three of them are putative α/β-hydrolases and precisely A0671 and B1632 have a P(3HB) synthase/depolymerase signature. Patatin-like phospholipase is a novel GAP (A0225) found in *R. eutropha* H16 that has not been described for binding to PHB granules of any PHB-accumulating species. Another GAP (A2001) with unknown function was related with P(3HB) metabolism since its encoding gene belongs to an operon with *phaB2* (acetoacetyl- CoA reductase) and *phaC2* (PHB synthase) [68].

A class of enzymes reported for the first time, found to be strongly associated and operating at the PHAs granules isolated from the thermophilic bacterium *Thermus thermophilus* cells grown in the presence of sodium gluconate. This class contains specialized lytic transglycosylases (LTGs), which are muramidases able to locally degrade the peptidoglycan (PG) meshwork of Gram-negative bacteria. Overall, a possible explanation concerning the localization of enzymes hydrolyzing PG on the surface of

PHA granules was hypothesized, that PHAs granules biogenesis may originate from membranes in accordance with a similar position recorded in the "budding model." The possible role of these enzymes may be probably "the promotion of septal PG splitting during daughter cell segregation" [69].

5.3. The Role of PhaP, PhaR, PhaF and PhaM Proteins in PHA Biosynthesis

Bacterial PHAs biosynthesis is a complex process requiring several enzymes and is affected by the mediation of proteins other than the principal enzymes such as PHA polymerase, or thiolase. Two proteins were extensively reported to implicate in PHAs biosynthesis, PhaP and PhaR proteins.

5.3.1. Phasins or PhaPs Proteins

Phasins, or PhaPs are low molecular weight proteins containing a hydrophobic domain associated with the PHAs, and a hydrophilic/amphiphilic domain exposed to the cytoplasm [70]. For many years due to the lack of the structural knowledge, the molecular basis for the various functions of the PhaPs had not been fully understood. They are proposed to play a significant role in PHA biosynthesis and granules generation since their function is both structural and regulatory and can influence PHA accumulation the number and size of the granules.

Their name is analog to oleosins - proteins on the surface of oil globules found in oleaceous plants [67]. They are the most abundant proteins in the PHA carbonosome [37]. Based on their sequence, phasins are distributed in four families (http://pfam. xfam.org/), namely PF05597, PF09602, PF09650 and PF09361. Phylogenetically phasins and phasin-like proteins constitutes a heterogeneous group of proteins, which contains a leucine-zipper motif [71].

A phasin from *Azotobacter* sp. FA-8 (AzPhaP) was demonstrated to hinder thermal aggregation *in vitro* of the citrate synthase used as a model protein and to expedite the refolding process of this enzyme which it was previously had been subjected to chemical denaturation [72]. Thus, AzPhaP exhibits chaperone-like functions both *in vitro* and *in vivo* in *E. coli* recombinants and suggests that phasins generally could possess a protective role in natural PHAs producer microorganisms.

The role of PhaP of *R. eutropha* has been extensively investigated. Generation of an *R. eutropha phaP* deletion strain demonstrated that PhaP must accumulate to high levels for playing its normal role in P(3HB) synthesis, whereas accumulation of PhaP to low levels is functionally equivalent to its absence. PhaP positively influences P(3HB) synthesis under growth conditions, which advance the formation of P(3HB) to low, intermediate, or high levels that generally go in parallel with these of PhaP. It was demonstrated that PhaP exhorts P(3HB) synthesis through regulation of the

surface/volume ratio of PHB granules, or interaction with PHA synthase and concluded that PhaP plays a significant role in P(3HB) synthesis even from the early stages in P(3HB) formation and under a range of growth conditions [73].

PHA biosynthesis also occurs in Cyanobacteria, which are photoautotrophic microorganisms, able to incorporate atmospheric carbon dioxide into carbon backbones via the Calvin-Benson cycle. Some cyanobacterial species like *Synechocystis* sp. strain PCC 6803 accumulate P(3HB) when essential nutrients elements like phosphorus or nitrogen are absent. The three principal enzymes found to participate in the P(3HB) biosynthesis namely, PhaA, PhaB, and the heterodimeric P(3HB) synthase PhaEC in this strain. Secondary-structure prediction and circular dichroism (CD) spectroscopy of PhaP in this strain divulged that the protein is composed of two α-helices connected to PHB granules, and purified PhaP forms oligomeric structures in solution in which both α-helices participate. The cyanobacterial phasin PhaP also found encoded by *ssl2501* and translational fusion of this protein with increased green fluorescent protein (eGFP) showed a clear co-localization to surface of PHB granules. Deletion of *ssl2501* diminished the number of PHB granules per cell, the average PHB granule size enhanced as it was expected for a representative phasin. Moreover despite the lack of any effect on the amount of PHB due to this deletion the biosynthetic activity of PHB synthase was negatively influenced. Conclusively, *ssl2501* encodes a cyanobacterial phasin, PhaP, which is able to regulate the ratio of surface-to-volume to PHB granules [74].

Halomonas spp. due to their ability to grow under unusual conditions of high pH and salinity has been employed as a host strain for low-cost production of PHAs. Specifically, *Halomonas* strain TD was utilized for high yield expression of PhaP and its development into bio-surfactant for industrial application in an economical way [75].

The crystal structure of PhaP from *Aeromonas hydrophila* (AhPhaP), was divulged to be a tetramer with 8 α-helices adopting a coiled-coil structure where each AhPhaP monomer exhibit both a hydrophobic and a hydrophilic surface, providing the surfactant properties. The discovery of the first PhaP crystal structure constitutes a significant contribution to the understanding of the mechanism of PHAs formation *in vivo* and of the reason of PhaP unique surfactant properties as well as to the development of their biotechnological applications as bio-surfactants and amphipathic coatings materials [76]. In spite of their considerable variability, only the crystal structure of AhPhaP has been solved to date and homology models were established with other phasins for rational mutagenesis [77].

The heterologous expression of phasin PhaP from the soil bacterium *Azotobacter* sp. strain FA8 in *E. coli* displayed a novel chaperone-like property to protect recombinant *E. coli* against several kinds of stress such as ethanol, butanol, and 1,3-propanediol. PhaP promotes growth and P(3HB) synthesis in polyester-producing recombinant strains and decreases the inclusion bodies formation during overproduction of heterologous proteins. These results recommend a novel application to the already multifunctional phasins

protein family, suggesting that expression of these proteins or other chaperones can be employed to ameliorate the production of biofuels and other chemicals [72].

Phasins have been remarked to influence both PHA accumulation and employment. Aside from their structural role in PHA granule, phasins possess a considerable variety of supplementary functions. Different phasins have been ascertained to (i) enhance PHA depolymerization, (ii) amplify both the expression and activity of PHA synthases, (iii) involve in PHA granule segregation and (iv) exhibit chaperone-like activities *in vivo* and *in vitro*. All these functions of phasins establish their role in PHA-related stress protection and fitness. Owing to their PHA granule binding ability and structural pliability, various biotechnological applications have been emerged employing different phasins, amplifying the scientific focus in the study of these remarkable proteins [78].

5.3.2. PhaF Phasin

Although the significance of phasins in PHA metabolism and regulation is recognized, a limited number of reports focused on their structure. Maestro et al. investigated the structure and stability of PhaF from *P. putida* KT2440, a protein implicated in PHA granule stabilization and distribution in the daughter cells upon cell division [71]. A leucine-zipper motif was found in their structure, indicating that formation of oligomers is an ordinary organization mechanism in these proteins [71]. Enzymatic activities, such as PG hydrolases, localized in PHAs granules were reported, suggesting that they promote septal PG splitting and daughter cell separation [69].

So far, four PHB-associated proteins with multiple locations and twelve proteins with PHB-specific locations were identified in a PHB granule fraction isolated from *R. eutropha*. These proteins are not present in the soluble, the membrane, and the membrane-associated fractions, whereas their function remains unknown for most of them. A putative α/β-hydrolase activity was attributed to three of them, and specifically A0671 and B1632 have a P(3HB) synthase/depolymerase signature. The third one, A0225, is a patatin-like phospholipase, and it has not been described before for any PHB-accumulating species. The fourth protein (A2001) is unknown although its encoding gene forms an operon with *phaB2* (acetoacetyl- CoA reductase) and *phaC2* (PHB synthase). Deletion of A0671 and B1632 encoding genes affected the P(3HB) mobilization ability of cells in the stationary growth phase, confirming their involvement in P(3HB) metabolism.

5.3.3. PhaR Protein

PhaR is a PHA granule-associated protein acting as a regulator in the PHAs biosynthesis in several bacteria. *R. eutropha* strains with deletions in *phaR*, *phaC*, and/or *phaP* genes were constructed and PhaP accumulation was monitored by immunoblotting. Both the *phaR* and the *phaR-phaC* deletion strains accumulated PhaP to higher levels than the wild type, which accumulated PhaP in a PHA production-dependent manner,

while no PhaP was accumulated in the *phaC* deletion strain. This finding denotes that PhaR constitutes a negative regulator of PhaP accumulation, the regulatory role of which is to specifically impede PhaP from accumulating in cells that are not producing PHA. Heterologous expression of the *R. eutropha phaR*, *phaP*, and PHA biosynthetic genes (*phaCAB*) into recombinant *E. coli* was adequate to functionalize the PhaR/PhaP regulatory system, signifying that PhaR is able to regulate both PhaP accumulation and directly PHA responds. Deletion of *phaR* resulted in a reduction of PHA yield, and a *phaR-phaP* deletion strain displayed a more pronounced PHA deficiency than a *phaP* deletion strain, signifying that PhaR advances PHA production and performs this at least partially via a PhaP-independent manner. Models concerning the regulatory roles of PhaR in PhaP and boosting PHA production are also presented [79].

Recently five genes encoding PhaP group proteins and one *phaR* gene have been identified in the genome of *Burkholderia* symbiont strain RPE75. As found in other bacteria the role of PhaR is the negative regulation of PhaP biosynthesis and PhaP proteins are surface proteins of PHA granules. The consequences of colonization capability in the host midguts and the appropriateness of host insects after providing *Burkholderia* mutant cells (four phaP-deleted mutants and one phaR-deleted mutant) to the host insects were reported. The interesting is that PhaR plays a remarkable role in the PHA granules biosynthesis and that it is significantly related to the colonization of the *Burkholderia* gut symbiont in the host insects' midgut. In other words, the study also supports the concept that the environment of the host midgut may not be propitious to symbiotic *Burkholderia* cells and that PHA granules may be necessary for adapting to the host midgut [80].

PhaR was also found in haloarchaea. The haloarchaeal homologs of the PhaR comprise a novel conserved domain, a “swapped-hairpin barrel” fold observed in AbrB-like proteins. Amino acid substitution in this AbrB-like domain pointed out that it is crucial for the repressional activity of PhaR. Specifically in *Haloferax mediterranei* PhaR and PhaP encoding genes are located in the same operon (*phaRP*) and their expression is negatively regulated by PhaR [81]. Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assays demonstrated a significant interaction between PhaR and the *phaRP* promoter *in vivo*, while a specific *cis*-element was revealed by scanning mutagenesis of the *phaRP* promoter as the feasible binding site of the PhaR. Furthermore, the *phaRP* promoter had a feebler activity in the PHA-negative strains, denoting an operation of the PHA granules in the titration of the PhaR. The absence of *phaR* in *H. mediterranei* strain resulted in deficient PHA accumulation and the produced granules were of irregular shapes. Interesting enough, the PhaR by itself can advance PHA synthesis and granule formation in a PhaP-independent way. Collectively the haloarchaeal PhaR demonstrated to be a novel bifunctional protein regulating PHA accumulation and granule formation in *H. mediterranei* [81].

5.3.4. PhaM Protein

Another multifunctional protein from *R. eutropha* H16 (RePhaM) was reported to be involved in PHAs biosynthesis. PhaM (26 kDa) from *R. eutropha* H16 was considered as a multifunctional GAPs including among its function (i) PhaM binds to PHB granules *in vivo* directly or indirectly through PhaC1 or/and PhaP5 thus exhibiting phasin-like properties. PhaM also defines the number and the ratio surface to volume of PHB granules produced, in a similar manner to phasins of other PHB-accumulating bacteria as PhaP1 or other [67, 82, 83]. (ii) Most remarkably, PhaM is a distinct phasin since turned out to be able to bind to DNA *in vitro* and to the nucleoid region *in vivo*, ending to be able to define localization of PHB granules by its properties of PHB- granule-binding and nucleoid-binding ability. Finally (iii) PhaM assists in the distribution of the generated PHB granules to both daughter cells in the course of cell division. In *Pseudomonas putida*, phasin protein PhaF [84, 85] exhibited DNA-binding ability [86] and was regarded important for the distribution of PHAMCL granules throughout cell division. Taken into consideration the suggested property of PhaM from *R. eutropha* H16 to involve in the PHA granules distribution to the daughter cells during cellular division [82], in combination together with the new enzymatic activities, specialized lytic transglycosylases (LTGs), peptidoglycan hydrolases that promote septal PG splitting and daughter cell separation [69], localized on PHA granules as GAPs, we directed to the hypothetic model (Figure 5). A number of lytic activities were detected PHA granules-associated but the main proteins have at 32 and 110 kDa.

PHB granules were attached to the nucleoid through the C-terminal PAKKA motif of RePhaM [87]. Additionally, RePhaM constitutes and operates as the physiological activator of P(3HB) synthase (PhaC1) [59].

It is interesting that RePhaM could activate *in vitro* PHA synthase from *R. eutropha* (RePhaC) but not PHA synthase from *Aeromonas caviae* (AcPhaC), whereas the protein interacted directly with RePhaC but not with AcPhaC. It is also notable that RePhaM has minor or indirect interaction with the PHA polymeric chain. PHA biosynthetic genes (*RephaA*, *RephaB*, and *RephaC/AcphaC*) as well as and the R*ephaM* gene were transferred into recombinant *E. coli* and were cultivated for PHA production. Unexpectedly upon the expression of RePhaM, PHA accumulation decreased and the morphology of PHA granules was altered in RePhaC-expressing *E. coli*. No variation neither in the amount of P(3HB) nor the morphology of PHA granules was observed by RePhaM expression in AcPhaC-expressing *E. coli* [88].

The activation of RePhaC1 by RePhaM was recently studied in details [44]. Protein pull-down experiments demonstrated that the N-terminal domain of PHA synthase (RePhaC1ND) is necessary for binding RePhaMΔC and RePhaMF. Thus, the formation of a complex between RePhaC1and RePhaM is mediated by the interaction between RePhaC1ND and RePhaM (RePhaMΔC and RePhaMF), which is considered responsible for the increase of RePhaC1 activity upon attachment to RePhaM. Further experiments

demonstrated that the complex is formed according to a 2:2 stoichiometry, as shown in Figure 6. One molecule RePhaMΔC is bound to each side of the dimeric form of RePhaC1F leading to the formation of an extended structure with the N-terminal domain of PHA synthase (RePhaC1ND). It was thus proposed that RePhaM activated RePhaC1 by extending the structure of RePhaC1ND strengthening thus the binding ability of RePhaC1ND to the progressing P(3HB) polymer [44].

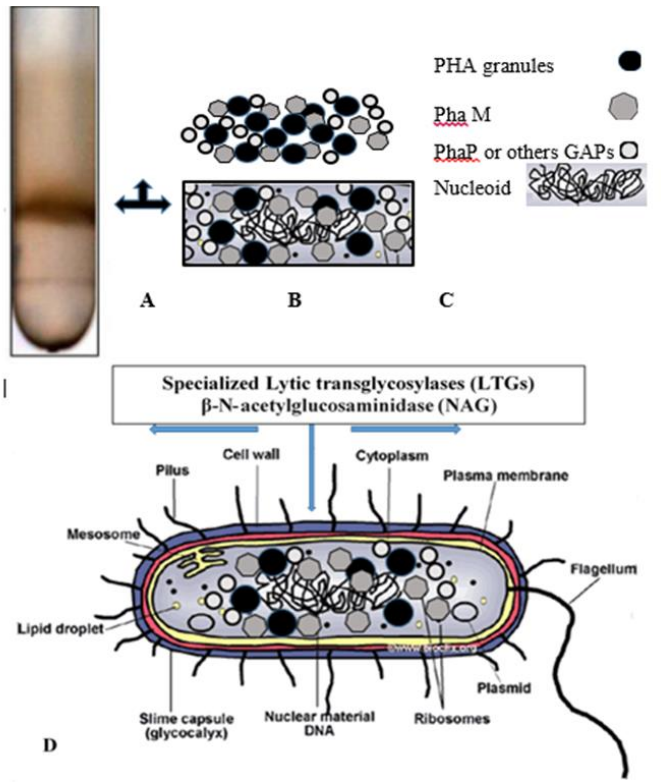


Figure 5. A. Discontinuous glycerol gradient for *T. thermophilus* PHAs granules purification. The arrows indicate the position of the PHAs granule's layer B and C. PHA granules-associated (PhaM, PhaP and other proteins) in the absence or presence of nucleoid.

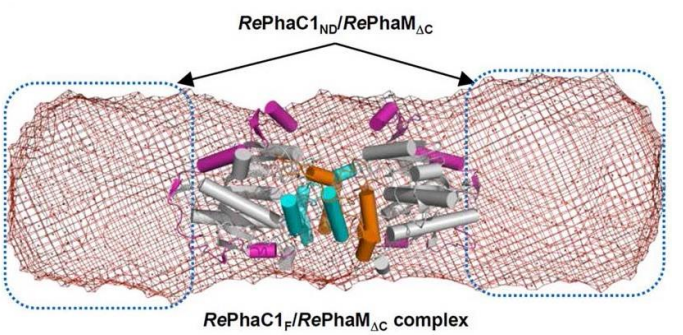


Figure 6. Reconstructed 3D structure of the RePhaC1F/RePhaMΔC complex. RePhaC1ND/RePhaMΔC part is shown as blue-colored dotted rectangles. Figure is reproduced from [44] after permission.

6. PHA BIOSYNTHESIS BY ENGINEERED BACTERIA

PHAs have been reported as biodegradable polymers with a wide range of applications and a high-cost production. One of the approaches to reduce cost is the construction of a super PHA producer strain, or by using cheap substrates such as molasses, sugar, and starch, or using transgenic plants, or by enhancement of the product yields. Another approach would be the construction of a microbial microorganism which is able to synthesize specific PHA polymers. To achieve a high PHA concentration several methods can be applied such as induction of PHA synthesis by O_2 limitation, by placing PHA synthesis operons behind microaerobic promoter, enlarging PHA production cells (by engineering cell division patterns and/or cytoskeleton), inducing cell flocculation or lysis (by inducible expression of surface displaying adhesive proteins or cell lysis proteins), hyper production of PHAs by manipulating PHA synthesis mechanism and PHA synthases or formation of PHA granules associated proteins and creating a synthetic cell that combines the above properties [89–91].

High levels of PHA productivity per dry cell weight and per time, unit and volume with the least cost are desirable characteristics for industrial PHA production [92, 93]. Recombinant bacteria are considered to have great potential for high-efficiency PHA production. In natural PHA producers, the addition of extra *phb* genes copies did not significantly increase the production [21, 94]. Genetic engineering of *E. coli* results in the production of PHAs fast and efficiently in comparison to natural PHA producers [95]. In addition, *E. coli* is a considerable candidate for PHA synthesis as PHAs can be easily purified and there are no PHA degrading enzymes. To synthesize PHAs in *E. coli* at least a PHA polymerase is required. This enzyme is critical for the 3-hydro acyl-CoA attachment to the already synthesized polymer. In the study of Rhie et al. [96] *E.coli* JMU193 were transformed with plasmids harboring a PHA polymerase gene (phaC1) from *Pseudomonas oleovorans* and a cytoplasm located thioesterase I gene (*tesA*) from *E. coli*. The transformed strain was enabled to store mcl PHAs when gluconate was used as a sole carbon source. The *E. coli* strain used had a deficient β-oxidation function, while gene expression was inducible and regulated. A 2.3% per cell dry weight of PHA accumulation was observed compared to the positive control of 15% accumulation by *P. putida* KT2442 grown in gluconate. The monomers that were produced are 3-hydroxyhexanoate, 3-hydroxyoctanoate and 3-hydroxydecanoate. The function of thioesterase I is to produce increased levels of free fatty acids [97] by acyl-ACPs hydrolysis leading the fatty acids to β-oxidation or PHA biosynthesis. However, in *E. coli* mutants that were used β-oxidation cycle was incorporated so all the produced free fatty acids were used exclusively for the PHA synthesis. It was also hypothesized that the recombinant *E. coli* accumulate PHAs as a result of *de novo* fatty acid synthesis and that there is a linkage of several steps of β-oxidation with thioesterase I [98].

To synthesize P(3HB-3HV) in *E. coli* an endogenous metabolic alteration was conducted. Since propionic acid is required for 3HV production which cannot be imported physiologically to *E. coli*, *ato* genes were introduced and overexpressed. *Ato* genes are responsible for acetoacetate and propionate import [99]. In addition, *fad* regulon which included a thiolase gene was inserted to fully degrade fatty acids to produce P(3HB-3HV) [15, 100, 101]. In the study of Theodorou et al. [99] it is reported that AtoSC two-components system upregulates the high-molecular-weight P(3HB) biosynthesis in recombinant *E. coli* strains transformed with the *phaCAB* operon of *Cupriavidus necator*. Additionally the copolymer P(3HB-co-3HV) biosynthesis in *phaCAB* + *E. coli* was unregulated. The AtoSC induction with acetoacetate resulted to 1.27 g/l production of P(3HB-co-3HV) with a 3HV fraction of 25.5% wt. and biopolymer content of 75% w/w in a time-dependent process [99].

Clustered Regularly Interspace Short Palindromic Repeats interference (CRISPRi) is a new method for specific gene regulation. The aim was to increase PHA synthesis by engineering *Halomonas* sp. TD01 using CRISPRi to regulate gene expression of *ftsZ*, *prpC* and *gltA* genes. FtsZ protein is involved in cell division by assembling the Z ring. Inhibition of *ftsZ* leads to filamentous cell forms and repression of cell mitosis [102, 103]. The *prpC* gene encodes a 2-methyl citrate synthase which catalyzes the formation of 2-methylcitrate. The latter substrate originates from propionyl-CoA [104, 105]. Finally, the *gltA* gene encodes the citrate synthase. Repression of *gltA* decreases acetyl-CoA consumption by the TCA cycle and consequently improves P(3HB) synthesis [106, 107]. Copolymer P(3HBV) and P(3HB) were synthesized by the genetically engineered *Halomonas* which proved the CRISPRi feasibility [108].

PHA biosynthesis includes many pathways as mentioned above. However, engineering microorganisms with large gene clusters remain cost-effective and difficult to handle. A new method has been developed for PHA biosynthetic genes insertion named single-strand overlapping annealing (SSOA). In this method, the target gene cluster is digested and the DNA fragments are recovered. Consequently, the single-strand DNA overhangs are specifically annealed and covalently attached to a circular and linear vector, respectively. This method enabled an 18 kb DNA fragment cloning [109]. Additionally, this method enabled the co-production of hydrogen and P(3HB) in *E. coli*, preventing toxic compounds formation such as formate [110]. Another method was developed based on Nicking Endonucleases for DNA ligation-independent cloning (NE-LIC) to generate 3′-end and 5′-end ssDNA overlaps to assemble a six genes pathway for microbial PHB biosynthesis [111].

A recombinant *E. coli* producing P(3HP) and P(4HB) [112] was further engineered by insertion of a synthetic pathway consisting of five genes encoding a 4-hydroxybutyrate-CoA transferase from *Clostridium klyuveri*, the ACS domain of propionyl-CoA ligase from *Chloroplexus aurantiacus* (domain with high similarity to acetyl CoA synthase), the dehydratase and the aldehyde dehydrogenase from *P. putida*

and a PHA synthase from *R. eutropha* to synthesize P(3HP-*co*-4HB) [113]. Additionally for P(3HB-*co*-4HB) synthesis in *E. coli*, a gene encoding succinate semi-aldehyde dehydrogenase was expressed under the control of CRISPRi [114].

An approach for high throughput P(3HB) synthesis from *phaCAB* operon of *R. eutropha* in *E. coli* was conducted. Rational designed ribosomal binding sites libraries with defined strength for each of the three genes were built. A 92% P(3HB) per dry cell weight was attained [115]. Wang et al. designed a metabolic pathway for P(3HB-*co*-HHx) synthesis by combining the β-ketothiolase-dependent condensation pathway with inverted β-oxidation cycle [116]. Zhuang et al. [117] synthesized mcl-PHAs in *E. coli* from glucose by engineering the reverse β-oxidation pathway [106, 117]. β-oxidation pathway and fatty acids synthesis are crucial for mcl-PHAs synthesis. When fatty acids used as a carbon source β-oxidation pathway plays the main role in the PHA biosynthesis. When a fatty acid enters the β-oxidation and loses in each cycle two carbon atoms shortened *n*-alkanoic acid monomers are provided for the PHA biosynthesis. To avoid the production of shortened PHA monomers *fadB* and *fadA* genes are deleted, which encode a 3-hydro acyl-CoA dehydrogenase and a β-ketothiolase, respectively, leading to the exclusive synthesis of HDD by *P. putida* [118–122]. A similar approach was followed for *P. entomophila* L48. In addition, PSEEN 00664 gene encoding an acetyl-CoA transferase was deleted. A *pha*-operon knockout mutant of *P. entomophila* L48 referred as *P. entomophila* LAC01 was constructed for PHA synthase specificity study [123].

Recently Anjum et al. [3] produced successfully a wide range of mcl-PHA homopolymers ranging from poly(3-hydroxyheptanoate) to poly(3-hydroxy-tetradecanoate), using the β-oxidation pathways of engineered *Pseudomonas entomophila* LAC23 grown in the presence of various fatty acids. In addition, random copolymers of 3-hydroxyoctanoate (3HO) and 3-hydroxydodecanoate (3HDD) or 3-hydroxy-tetradecanoates were produced, and their constitution could be monitored by regulating the proportions of two related fatty acids. Meanwhile, random and block copolymer P(3HO)-b-P(3HDD) was synthesized by the same strain in the study of Wang et al. [122]. P(3HO-*co*-3HDD) were synthesized from engineered *P. entomophila* LAC23, deficient in β-oxidation, when sodium octanoate and dodecanoic acid were supplemented. The monomer composition could be controlled by regulating the ratio of the two carbon sources. Also, block copolymers P(3HO)-*co*-P(3HDD) were produced when sodium octanoate and dodecanoic acid were gradually added to the cultures. Engineered *P. entomophila* LAC01 with *pha*-operon knockout and harboring PHA synthase gene phaC from *Aeromonas hydrophila* 4AK4, was able to synthesize scl- and mcl-PHAs random copolymer P(3HB-*co*-3HHx) using dodecanoic acid [122].

Finally, *Halomonas* bacteria were reconstructed to generate PHA in modifying morphology for inexpensive production and under non-sterile and uninterrupted

conditions. It is expected that the synthetic biology will progress the PHA into an industry of bio-materials [124].

Recent achievements demonstrated the construction of a microbial platform in order to produce not only accidental copolymers with controllable monomers and their proportions but additionally specified structurally homopolymers and block copolymers. This purpose was succeeded by engineering the genome of *Pseudomonas putida* or *Pseudomonas entomophiles* to attenuate the biosynthetic pathways of β-oxidation and *in situ* fatty acids, so that a fatty acid provided to the bacteria retains its original chain length and structures upon its incorporation into the PHA chains. The engineered bacterium permits functional groups in a fatty acid to be incorporated into PHAs, constituting liturgical PHAs, which, upon grafting, produces endless PHA diversity. Efficient production of the most powerful member of PHA the poly(3-hydroxypropionate) or P(3HP), succeeded also by recombinant *E coli.* Synthetic pathways of P(3HP) and its copolymer P(3HB-*co*-3HP) of 3-HB and 3-hydroxypropionate were congregated, respectively to permit their synthesis from glucose. An advanced CRISPRi was also successfully employed to operate simultaneously multiple genes and monitor the metabolic flow in *E. coli* to achieve a range of copolymer P(3HB-*co*-4HB) of 3HB and 4HB. Moreover, the bacterial shapes were successfully engineered for increased PHA production [125].

The highest rate of PHAs is produced from bacteria and archaea which is considered to provide increased survival in stress environments. However, it has been reported PHA synthesis in transgenic plants [5,126] such as *Arabidopsis thaliana* [127], tobacco [128] and rape [129] and other eukaryotes such as human where P(3HB) is a cellular membrane component [130]. Prokaryotic PHAs rather than eukaryotes PHAs are mostly used for various applications. In addition, the main PHA reported to be synthesized from bacteria is P(3HB) [5].

The study of O'Byrne et al. [131] was focused on identifying factors enhancing P(4HB) production in recombinant *E. coli* strain. Among PHAs, P(4HB) acquires much attention due to its biodegradability, biocompatibility, and medical applications. Reports for P(4HB) synthesis as well factors affecting accumulation remain scarce. It was found that addition of propionic acid enhances P(4HB) production while external methionine suppresses it. Recombinant *E. coli* JM109 harboring plasmid that contains the *phbC* gene (PHA polymerase) from *R. eutropha* and 4-HB-CoA transferase from *Clostridiym kluyveri* was used. The latter enzyme facilitates P(4HB) production in presence of 4HB. Different media were used to identify which are more efficient for P(4HB) production. A 61% (w/w) and 30% (w/w) of P(4HB) accumulation was observed in modified E2 medium and M9 respectively in combination with reduced growth rate. This suggested an implication of NZ-amines (amino acids and peptides produced by casein hydrolysis) in P(4HB) accumulation. An explanation was proposed: P(4HB) is generated from 4HB and acetyl-CoA. Since acetyl-CoA is involved in random metabolic pathways such as energy

production and amino acid synthesis, when NZ-amines were provided in the medium, more acetyl-CoA was available for P(4HB) production. However, limitation of certain amino acids pools could promote P(4HB) synthesis by limiting cell growth. Specifically, it was stated that addition of propionic acid limits methionine storage and cell growth [131]. Addition of propionic acid did not affect P(4HB) of recombinant *E. coli* when grown on glucose. However, when glycerol was used as carbon source, the inclusion of propionic acid derived in 43% (w/w) of P(4HB) production in comparison 17% (w/w) without propionic acid. The stimulating effect of propionic acid was decreased by the inclusion of extracellular methionine (30% w/w P(4HB) formation). This observation suggested the existence of a mechanism where propionic acid decreases methionine pool resulting in more P(4HB) formation. Also it was proposed that 2 g/L of propionic acid is the best concentration for P(4HB) production (80% (w/w) P(4HB) synthesis under experimental conditions) [132].

The three-step biosynthesis pathway genes of P(3HB) production were identified in *Caldimonas manganoxidas* consisting of the *phaCAB* genes. In order to investigate the functionality of the pathway, the three genes were cloned into *E. coli* strain proving the P(3HB) production. In addition, it was found that PHB synthase of *C. manganoxidans* appeared to have a 60% similarity to existing class I PHA synthases. A wide variety of microorganisms produce P(3HB). Among thermophilic strains, *C. manganoxidans* demonstrates the highest performance. Cloning and overexpression of *M. manganoxidans* genes in *E. coli* BL21(DE3) strain indicate a potential use in industrial P(3HB) production. Transformation of *E. coli* with plasmids harboring *phaABC* genes demonstrated that the three-step pathway can be reconstructed successfully. In addition, it was shown that PhaB obtains a significant effect on P(3HB) production while increased levels of PhaC does not affect it. Overexpression of *phaB* resulted in an increment of P(3HB) production. A hypothesis for this has not been established [35].

In the study of David et al. [133], four butyryl-CoA transferases were examined originated from *Roseburia* sp., *Eubacterium hallii*, *Faecalibacterium prausnitzii* and *Anaerostipes caccae* to enhance synthesis of PHAs containing 2-HA monomers such as lactic acid and 2-hydroxybutyrate. Previously, it was found that naturally produced PHA synthases consider 3-, 4-, 5-, 6- HAs monomers as substrates. Recombinant *E. coli* were produced that can assemble PHAs containing 2-HAs by engaging a butyryl-CoA transferase which provided 2-HAs-CoA, as well as an evolved and mutated PHA synthase, originated from *Pseudomonas* sp. strain which is able to polymerize 2-HAs-CoA into PHAs [133].

Fluorine constitutes an important element for synthetic molecules production used for medicinal, agricultural and material management. Thus, it is rarely found in natural metabolism. There has been interesting in engineering the biosynthetic machinery of the cells to produce organofluorine targets. A five-gene system in genetically modified *E.*

coli for the biosynthesis of 2-fluoro-3-hydroxybutyryl-CoA using fluoromalonate was constructed. A malonate transporter, malonate:CoA ligase, a malonyl-CoA transferase, a reductase and a PHB synthase from *R. eutropha*, were inserted in plasmids in order to synthesize a fluorine PHA polymer. Poly(FHB-*co*-HB) were successfully synthesized containing up to 15% of fluorine monomers. However, it was mentioned that PHA synthase did not discriminate the stereochemistry of 2-fluoro substituent which could lead to amorphous polymer constructions [134].

7. PHA BIOSYNTHESIS BY OTHER ORGANISMS

In order to reduce the cost of P(3HB) production there have been made efforts to produce PHAs in plant crops. Studies have reported PHA synthesis in yeast, insects and plant species. Researchers have managed to transfect *Trichoplusia ni* (cabbage looper) cells with *phbC* gene which encodes a PHA polymerase of *R. eutropha* and succeeded to produce an active amount of protein [135]. In addition, insect cells of *Spodoptera frugiperda* were co-transformed with a mutant fatty acid synthase which does not extend fatty acids beyond 3HB, and a PHA polymerase resulting in 0.16% of dry cell weight production of PHA [136]. Also, there have been achieved formation of P(3HB) in plants by overexpression of bacterial *phb* genes resulting in identical to the natural bacteria producers products [137]. Eukaryotic cells are characterized by compartmentalization. In order to produce high levels of PHAs targeted expression of *phb* genes in specific organelles with high acetyl-CoA concentration needs to be achieved. The first choice to produce P(3HB) from plants was the plant model *Arabidopsis thaliana*. Since physiologically it contains 3-ketoacyl-CoA thiolase Arabidopsis was transfected with the genes of reductase and PHA polymerase originating from *R. eutropha*. Low P(3HB) production yield and growth defects were observed [138]. Consequently, a more successful P(3HB) formation was postulated by targeted expression of P(3HB) biosynthesis genes in plastids resulting in 14% of dry cell weight production of the polymer in Arabidopsis leaves [139]. In addition, P(3HB) production was tested in *Gossypium hirsutum* (cotton) [140] and *Zea mays* L. (maize) [15, 141].

Cyanobacteria are able to produce PHAs naturally hence less than heterotrophic bacteria. Thus, genetic manipulation, new harvesting techniques and alternate economical carbon sources and specific inhibitors can potentially improve PHA accumulation. Some cyanobacteria are able to use CO_2 and light to synthesize P(3HB) going from Calvin-Bensson-Bassham cycle via glycolysis to pyruvate and then to acetyl-CoA and finally to P(3HB) [142]. In *Synechocystis* PCC 6803, transcription regulator factors, such as RNA polymerase sigma factor E (SigE) and response regulator (Rre37), have been found, whose overexpression resulted in increased PHA accumulation. SigE incremented the

transcription genes involved in pentose phosphate pathway and glycogenolysis and altered metabolic rate of Krebs cycle. Rre37 was identified as a pathway regulator affecting metabolic flow from glycogen to P(3HB) [143–145].

Only class III PHA synthases have been found in cyanobacteria species [40]. Cyanobacteria produce PHAs via oxygenic photosynthesis. Glycogenesis pathway competes with PHA biosynthesis for the glycogen synthesis at the level of 3-phosphoglycerate produced from CO_2 assimilation through Rubisco by the carboxylation of ribulose-1, 5-biphosphate [146]. Metabolic inhibitors leading to disproportion of C: N and NADPH: ATP ratios have the potential to lead toward increased P(3HB) production [147, 148].

8. Novel Production of PHA Polymers and Nanomaterials

A promising approach for polymer and nanomaterial production is the use of sustainable systems such as microorganisms. It has been reported that *Pseudomonas* strains can be used as cell factories for mcl PHA production [120, 149], thus it is metabolically versatile and can be used as bioremediation reactions [150]. Consequently, *Pseudomonas* strains have been accomplished to synthesize Cadmium-based Quantum dots (CdS QDots) [151]. QDots obtained many applications including drug-delivery platforms, biomedicine, and biotechnology. A more sustainable synthesis can be achieved in microorganisms compared to chemical production where it is highly costly and toxic solvents are used. A lot of attention was given for co-production of biopolymers and nanoparticles as a more cost-competitive option and their usage in cell targeting under *in vivo* conditions. The purpose was to co-synthesize mcl-PHAs and CdS Qdots in *Pseudomonas putida* KT2440 cultures. Firstly, PHAs were produced in cultures grown on minimal salt medium and then for nanoparticles production $CdCl_2$ was added after 48-68 h of cultivation. To induce Qdots production cysteine was added additionally. It was reported that $CdCl_2$ did not significantly affect the PHA production indicating a Cadmium resistance of *Pseudomonas*. Compounds such as cysteine, glutathione, and sulfide play a major role in Qdots formation [152]. Three mcl-PHA monomers were identified: 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate. Qdots and PHAs were pinpointed in different locations. PHAs were located in the cytoplasm as inclusion bodies while Qdots appeared in the periplasmic space. *Pseudomonas* showed increased Cadmium resistance and PHA production was not affected in coproduction case [153].

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Chapter 3

POLYHYDROXYALKANOATES: A SHORT TALE FROM POLYMER SYNTHESIS TO FUTURE PROSPECTS

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are bacterial polyesters belonging to the most important group of bio-based and biodegradable polymers. A large number of bacteria possesses the ability to synthesize PHAs as carbon energy storage and a variety of monomer constituents of PHAs has been described, whose chemical composition range from straight to aromatic structures. The PHA synthesis is strongly influenced by the carbon source utilized for microbial growth and the metabolic pathways involved in the polymer synthesis, which the PHA synthase plays an essential role in the PHA polymerization process. This chapter introduces PHAs from their biochemical concept to a promising alternative for petrochemical compounds as eco-friendly thermoplastics. Among many suitable characteristics for industrial applications the most important attribute of PHAs is their biodegradability. The challenges faced by the industry of biopolymers in order to scaling-up the PHA production and increase their economical

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feasibility in the global market, including recent advances to minimize costs and to improve the polymer yield, besides the sustainable production of PHAs from agro-industrial by-products are also a focus of this review chapter.

Keywords: polyhydroxyalkanoates, biopolymer, microbial polyesters, biodegradable polymer, eco-friendly thermoplastic

1. INTRODUCTION

Plastics are polymers and additives, which exhibit interesting properties such as hardness, thermal insulation, electrical isolation, resistance to heat, organic solvents, oxidation and microorganisms. According to their chemical structures, a wide variety of plastics can be obtained, resulting in endless applications for packaging, building materials, automotive industry, medical and pharmaceutical products, and many other market sectors [1]. About 50% of plastic products are intended to be disposable for short-lived applications [2-3]. Since these materials are typically derived from petrochemical compounds and so they are not biodegradable, the inappropriate discard and the environmental persistence of plastics have become a global issue [4]. Therefore, the plastic disposal is a major concern. The plastic footprint is considered more dangerous than carbon footprint. Only 10% of total plastic waste is recycled and over 60% of plastic materials is discarded in landfills after their initial use. Plastics are harmful to terrestrial and aquatic ecosystems. The North Pacific garbage patch has increased by 100-fold in the last 40 years [5], which has reached continental dimensions. All these plastics are fragmented to particles less than 5 mm, the microplastics, and are assimilated by aquatic food chains, and thus, many times end up in the human food nutrition. Hundreds of billions of plastic bags are produced each year for a minute useful life, with a significant fraction being discarded in the environment and consecutively harming the ecosystems [1].

Due to their versatile properties it is very difficult to reduce the plastic consumption. However, the bioplastics bring possibilities to replace the petrochemical-based plastics by alternative materials with polymer-like properties [4]. Not all bioplastics are bio-based or biodegradable. It is partially misleading to use the expression bioplastic to make a distinction from petroleum-based polymers [1]. There are bio-based bioplastics such as bio-PET (bio-based polyethylene terephthalate), commonly used to produce beverage bottles, which is obtained from renewable biomass, but is not biodegradable and neither ecologically friendly. On the other hand, polycaprolactone (PCL) is a petrochemical-based plastic which presents biodegradable properties [1, 6, 7]. Accordingly, a polymer derived from biomass is not necessarily biodegradable, and not always a petroleum-based polymer is non-biodegradable. Therefore, bioplastics are bio-based, biodegradable or

both [8]. They exhibit the same or similar properties as conventional plastics added to environmental benefits. If a bioplastic is derived from biomass it helps to reduce the carbon footprint, since the released CO_2 can be minimized by plant CO_2 consumption during photosynthesis. In addition, if a bioplastic is biodegradable it is available for organic recycling by microorganisms from terrestrial and aquatic ecosystems, reducing significantly its environmental persistence [1, 8].

The biodegradable plastics are a very interesting ecological alternative, especially regarding the short-lived and disposable plastics [1-3]. Biodegradability combined to composting properties bring an additional solution for waste stream management, which not only can be biodegraded but also produce a humus-rich soil [8-9]. Polyhydroxyalkanoates (PHAs) are a family of bioplastics which are bio-based and fully biodegradable. They are compostable and also meet the standard specification for marine degradability. In addition, PHAs are attractive for medical uses due to their biocompatible features (Figure 1). The physical and chemical characteristics of PHAs can make them resistant to grease and oils, besides boiling water [1]. PHAs have faced their main delay in their relative high production cost and so the PHA production capacity is still small. However, the PHA prices have increasingly dropped due to processing upgrades involving new purification and extraction methods besides the utilization of cheaper raw materials [4], which highlight that the establishment of the PHA market is only a matter of time. For this reason, the PHA producers and several companies are optimistic and see the PHAs as promising bioplastics. Since many investors are betting on PHA potential, the most dynamic development is foreseen for PHAs and their production capacity is expected to grow threefold by 2021 [10].

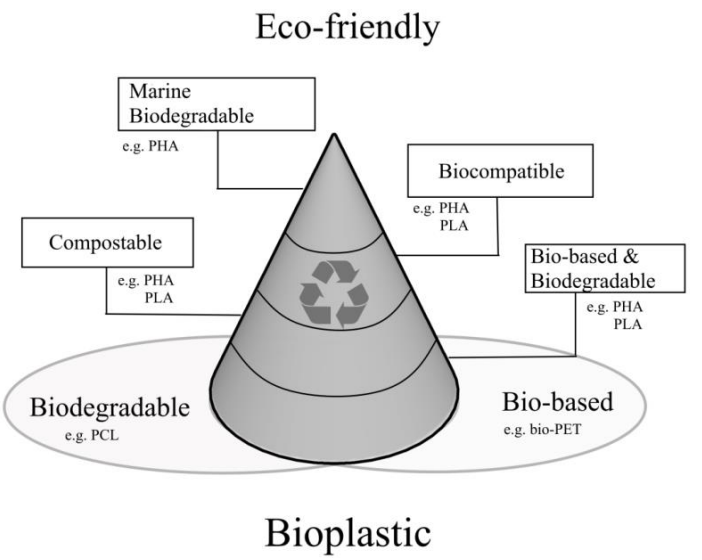

Figure 1. Bioplastics are bio-based and/or biodegradable plastics. PHAs belong to the family of bio-based and biodegradable plastics, they are also compostable, biocompatible and attend the standard specification for marine degradability.

This review chapter outlines the chemical structure, bacterial synthesis and applications of PHAs. Furthermore, this chapter approaches the sustainable production of PHAs from a variety of industrial by-products and recent advances in the pursuit of cost effectiveness. Finally, the challenges faced by PHA market are discussed as a focus on their future trends.

2. PHAs are Classified According to Their Chemical Structure

PHAs are bacterial polyesters accumulated as intracellular granules of energy storage under excess carbon source and nutrient imbalance (Figure 2). An average number of cytoplasmic inclusions of 8-14 has been observed with a diameter ranging from 0.24 to 0.50 μm for *Cupriavidus necator*, the most well-known PHA-producing bacterial strain. Light-scattering measurements of P(3HB) granules showed a molecular weight of 5 x 10^9 Da, while for the extracted polymer was observed a molecular weight ranging from 10^3 to 10^6 Da. The intracellular granules contain a minimum of 1,000 polymer chains and possess a membrane coat about 2 nm thick, whose PHA synthases and depolymerases are associated [11].The number and size of intracellular granules; chemical structure and physio-chemical properties; and the monomer composition are dependent of bacterial metabolism. The PHA monomers vary from 3 (C3) to over than 14 carbon atoms (C14). According to the content of monomer carbon atoms, PHAs can be classified into three broad classes: 1) short chain length PHAs (scl-PHAs) with monomers varying from C3 to C5; 2) medium chain length PHAs (mcl-PHAs), whose monomers range from C6 to C14; and 3) long chain lengh PHAs (lcl-PHAs), which are less common and studied, with monomers containing over C14 (Figure 3). scl-PHAs exhibit properties that resemble conventional thermoplastics while mcl-PHAs are similar to elastomers [12].

The poly(3-hydroxybutyrate) [P(3HB)] homopolymer is the most well-known and abundant example of scl-PHAs, which can be polymerized with other kind of monomers forming heteropolymers such as the copolymers poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] and poly(3-hydroxybutyrate-*co*-3-hydroxy-hexanoate) [P(3HB-*co*-3HHx)], and the terpolymer poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-4HB-*co*-3HV)]. Most of the bacterial strains synthesize scl-PHAs containing primarily P3HB while mcl-PHAs are mainly composed of 3-hydroxyoctanoate (HO) and 3-hydroxydecanoate (HD) monomers such as the mcl-PHAs produced by *Pseudomonas* strains [12, 14, 15]. More than 150 constituents of PHAs have been described as homopolymer or a combination of different monomers, which can be constituted of unsaturated bonds, cyclic compounds with aromatic or non-aromatic chemical structures, besides epoxides and halogens [16]. The chemical

composition of PHAs is directly linked to their properties. The P(3HB) is similar to petroleum-based polypropylene (PP) and offers good resistance to moisture. P(3HB) is brittle and stiff with elongation at break typically below 15% and Young's modulus (*E*) above 1 GPa. P(3HB) has been considered as a fragile material due to re-crystallization during storage showing decreasing values of elongation at break over time. Hence, these P(3HB) features have been improved by the use of plasticizers and nucleating agents and so reducing the crystallization process [17].

Like PP, the P3HB displays high melting temperature (*Tm* = 173-180°C) and relatively high tensile strength (30-40 MPa). However, the elongation at break of pure P(3HB) is approximately 5% while PP reaches 400%. Even the copolymer P(3HB-*co*-3HV) exhibits elongation at break lower than 15% and its modulus and fracture stress are 1.2 GPa and 25 MPa, respectively. The P(3HB) *E* modulus is 3.5-4 GPa and the glass transition temperature (*Tg*) of P(3HB) is close to room temperature (5-9°C) [18, 19]. On the other hand, mcl-PHAs have *Tg* values lower than room temperature, ranging from -65°C to -25°C. The *Tm* of mcl-PHAs ranges from 42°C to 65°C. Due to lower *Tg* values and degree of crystallinity the mcl-PHAs exhibit elastomeric properties and resemble natural rubbers. The *Tg* values of the mcl-PHAs decrease with the increase of the monomer chain length as a function of a higher mobility of the polymer chains. Therefore, mcl-PHAs containing C6-C8 monomers have higher *Tg* value (-25°C) than those formed by C8-C10 monomers (-40°C) [19]. In addition, the odd number fatty acids affect the structural arrangements of both backbone and side chains resulting in a different crystalline packing compared to even number fatty acids. *Pseudomonas aeruginosa* cultivations on both odd and even number fatty acids resulted in mcl-PHAs with lower *Tm* for polymers obtained from odd number carbon sources than those produced from even ones [20].

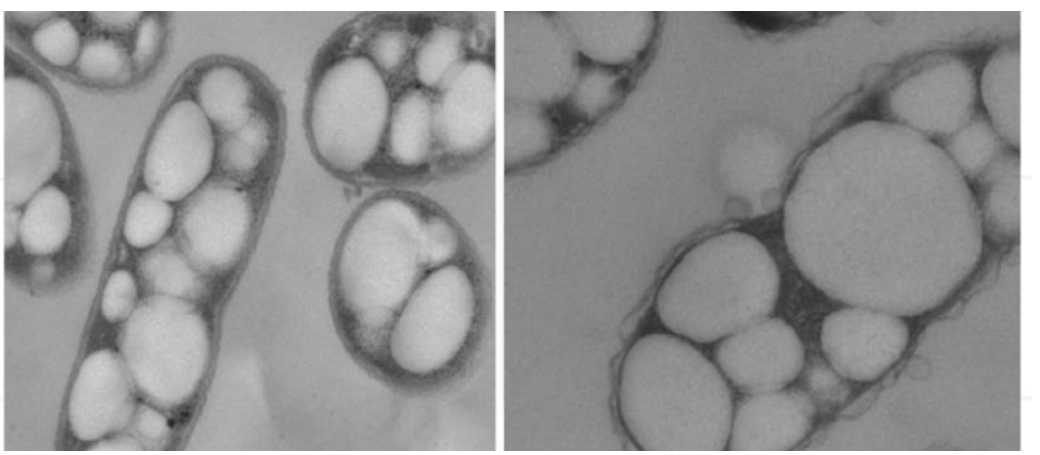

Figure 2. Bacterial cells containing PHA granules observed from transmission electron microscopy images (TEM) [13].

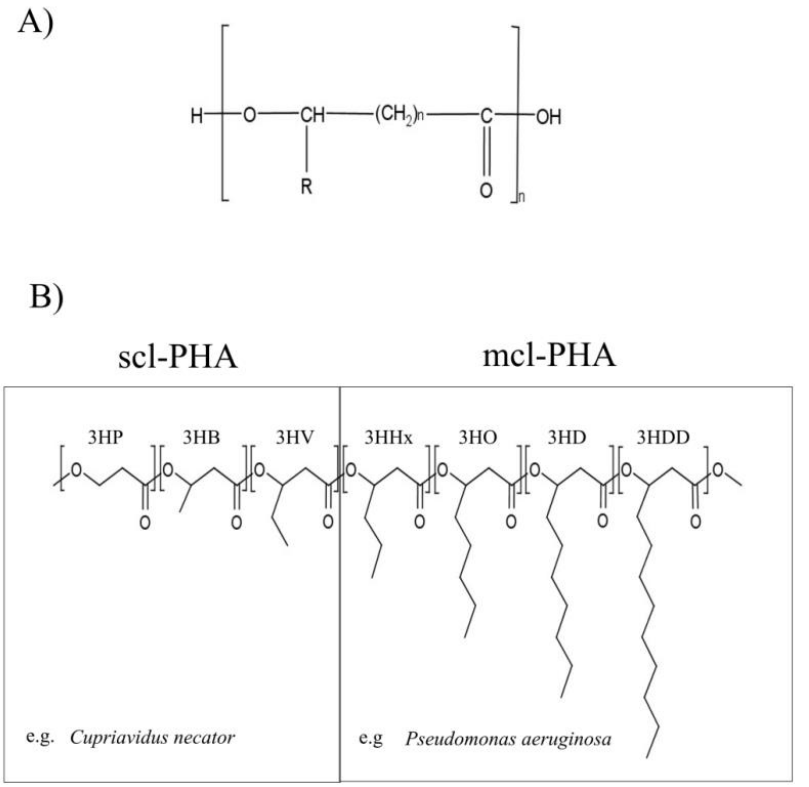

Figure 3. A) General PHA formula. B) scl-PHA monomers: 3-hydroxypropionate (3HP) (synthetic/non-natural), 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV); mcl-PHA monomers: 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD).

The molecular weights of mcl-PHAs are relatively lower than those of scl-PHAs and range from 40,000 to 412,000 Da [19]. The processability of PHAs has been improved by lowering the processing temperature and reducing the brittleness of scl-PHA based plastics, in particular P(3HB), which can be achieved by blending different PHAs [17]. The incorporation of HA monomers is a common strategy to improve P(3HB) properties. The P(3HB-*co*-3HV) copolymer obtained from the addition of 3HV units during the polymer synthesis can exhibit different thermomechanical properties as the 3HV composition varies in a range from 0 to 30 mol% [18]. Nevertheless, P(3HB-*co*-3HV) with high 3HV content are as brittle as the P(3HB) homopolymer [21]. The P(3HB-*co*-3HHx) resemble properties of the fossil-based polyethylene (PE). The terpolymerP(3HB-*co*-3HV-*co*-3HHx) containing 39 mol% of 3HV and 3 mol% of 3HHx showed improved properties with tensile strength of 12 MPa and elongation at break of 408%. Since poly(4-hydroxybutyrate) [P(4HB)] is more malleable than P(3HB), with a tensile strength compared to PE, the terpolymer P(3HB-*co*-4HB-*co*-3HV) containing 93 mol% of 4HB and 3 mol% of 3HV displays an elongation at break of 430%, toughness of 33 MPa and *E*

modulus of 127 MPa, which resembles PE properties; while the terpolymer containing 55 mol% of 4HB and 34 mol% of 3HV exhibited characteristics similar to PP [18].

3. THREE PATHWAYS ARE THE BASIS OF PHA BIOSYNTHESIS

The PHA synthesis is dependent to a great extent on the carbon sources that are supplied to the cultivation media. These carbon sources can be divided into PHA structurally related and unrelated carbon sources. The fatty acids are similar to hydroxyalkanoic acids in their structure and so they are related carbon sources. On the other hand, glucose is not similar to PHAs and is considered as non-related carbon source. According to the carbon sources utilized for bacterial cultivations there are three major pathways involved in the PHA synthesis [22] (Figure 4). The majority of prokaryotes synthesize P(3HB), the most well-known among PHAs with the most well-known and simple synthesis pathway. The P3HB formation requires the condensation of two acetyl-CoA molecules catalyzed by the β-ketothiolase, which is encoded by the PhaA gene, resulting in the formation of acetoacetyl-CoA. The latter is reduced to (R)-3-hydroxybutyryl-CoA by the (R)-3-hydroxybutyryl-CoA reductase (PhaB). Finally, the (R)-3-hydroxybutyryl-CoA is the direct precursor of P(3HB) synthesis, which is performed by the PHA synthase (PhaC). mcl-PHAs containing (R)-hydroxy fatty acids are synthesized by converting intermediates of fatty acid metabolism to (R)-hydroxyacyl-CoA. In the β-oxidation pathway the oxidation of enoyl-CoA to (R)-3-hydroxyacyl-CoA is catalized by (R)-specific enoyl-CoA hydratase (PhaJ). If the carbon source is oxidized to acetyl-CoA from unrelated carbon sources, excluding the fatty acid β-oxidation pathway, intermediates from the fatty acid *de novo* synthesis are transferred from their ACP (acyl carrier protein) thioester form to CoA by the specific transacylase (PhaG). Both fatty acid metabolic pathways have (R)-3-hydroxyacyl-CoA as the final substrate for the PHA synthase (PhaC) polymerization process [23].

As can be seen, the PHA synthase plays an essential role in the polymer production. According to the substrate specificity and the subunit composition, the PHA synthases can be divided into four major classes. The PHA synthases of class I and II comprise enzymes containing one subunit with molecular mass ranging from 61 kDa to 73 kDa. Class I PHA synthases utilize CoA thioesters of (R)-3-hydroxy fatty acids containing from 3 to 5 carbon atoms such as (R)-3-hydroxybutyryl-CoA, the substrate utilized by the PHA synthase of *C. necator* for P(3HB) biosynthesis. The class II comprise PHA synthases that utilize CoA thioesters of (R)-3-hydroxy fatty acids formed by 6 to 14 carbon atoms such as the PHA synthases observed in *P. aeruginosa* strains which are able to synthesize mcl-PHAs. Class III are consisted of PHA synthases containing two different subunits with approximately the same molecular masses (40 kDa). Whereas the PhaC subunit is similar to class I and II PHA synthases, the PhaE subunit is not similar to

PHA synthases. As the PHA synthases of class I, the class III PHA synthases utilize CoA thioesters of (R)-3-hydroxy fatty acids comprising 3 to 5 carbon atoms. *Allochromatium vinosum* is an example of bacterial species belonging to the class III PHA synthases. Enzymes of class IV resemble the class III PHA synthases. However, the PhaE subunit is replaced by PhaR with lower molecular mass of approximately 20 kDa. *Bacillus megaterium*, one of the first P(3HB)-producing bacterial species described in the literature, features class IV PHA synthases [24, 25]. *Thiocapsa pfennigii*, *Aeromonas punctata* and *Pseudomonas* sp. 61-3 are bacterial species whose PHA synthases are exceptions to the common classification. The *T. pfennigii* PHA synthase has a broad substrate specificity from CoA thioesters of 3 to 5 carbon atoms to 3-hydroxy fatty acids of 6 to 14 carbon atoms. Further, this synthase is consisted of two subunits with strong similarity to the PhaC of class III PHA synthases. Differently, the PHA synthase of *A. punctata* is formed by one subunit similar to PHA synthases of class I, which catalyses the production of a P(3HB-*co*-3HHx) copolymer. *Pseudomonas* sp. 61-3 presents a PHA synthase with high similarity to class II PHA synthases, but comprising two subunits, PhaC1 and PhaC2 [24].

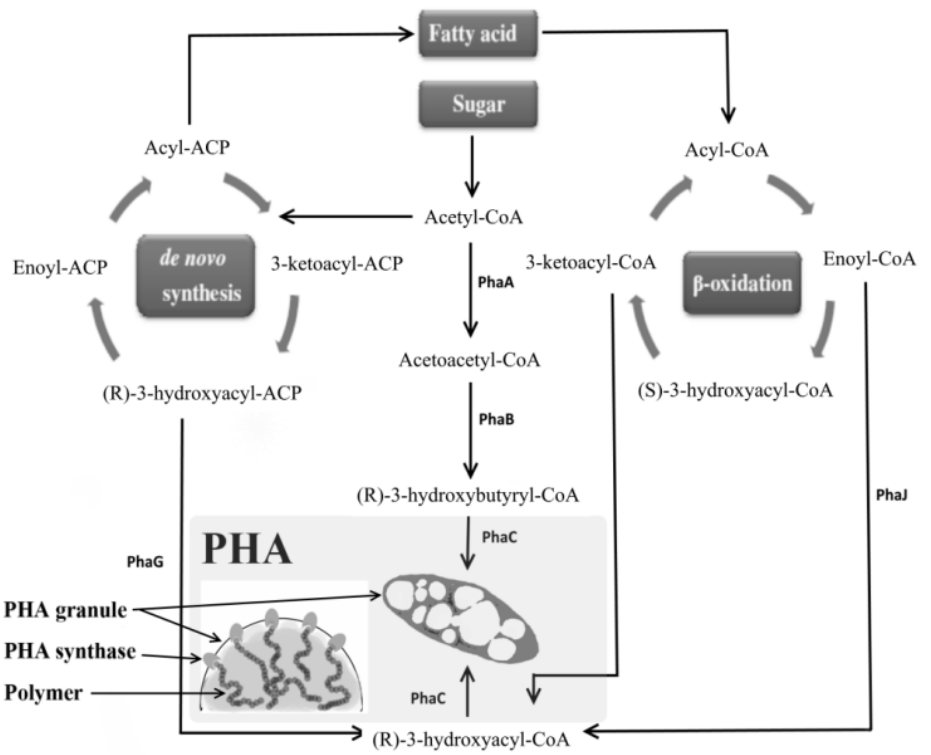

Figure 4. Three major PHA synthesis pathways.

The three basic biosynthesis pathways of PHAs can also be genetically engineered for diversifying and/or improving the PHA biosynthesis. About at least 12 pathways can

be generated from the classical PHA production pathways which are coupled with other native or artificial metabolic pathways resulting in a diversity of tailored PHAs. A variety of microorganisms has been employed in order to modify or create PHA metabolic routes towards to increase the polymer yields, reduce the production costs or even to introduce novel PHAs. Structurally related substrates such as propionate, valerate, hexanoate and 1,4-butanediol have been utilized to obtain PHA copolymers such as P(3HB-*co*-3HV), P(3HB-*co*-3HHx) and P(3HB-*co*-4HB). Since most of these carbon sources are toxic and/or expensive, many PHAs have been obtained from genetic engineering utilizing low-cost and non-toxic carbon sources including poly(3-hydroxybutyrate-*co*-lactide) [P(3HB-*co*-LA)], poly(3-hydroxybuty-rate-*co*-3-hydroxypropionate) [P(3HB-*co*-3HP)], besides the afore-mentioned copolymers which can be synthesized by the PHA synthase PhaC1 of *C. necator* due to its ability to polymerize scl-PHAs from different native substrates. The downregulation of the fatty acid β-oxidation pathway and the 3-hydroxyacyl-ACP-CoA transacylase (PhaG) are alternatives to enhance the flux of related fatty acids towards mcl-PHA accumulation [22]. Although several enzymes are involved in the fatty acid conversion into mcl-PHAs with a broad specificity for monomers consisted of 6 to 14 carbon atoms, an acyl-CoA ligase from *P. putida* (PP 0763) has been reported to act on (R)-3-hydroxy fatty acids derived from the fatty acid biosynthesis via (R)-3-hydroxyacyl-ACP-CoA thioesterase producing mcl-PHAs from glucose, an unrelated carbon source [26, 27]. The PhaG and PP0763 genes encoding the 3-hydroxyacyl-ACP-CoA transferase and acyl-CoA ligase, respectively, besides the PhaC1 gene which encodes the PHA synthase of *Pseudomonas* sp. 61-3, were cloned and co-expressed in *Escherichia coli* resulting in mcl-PHAs from glucose and so demonstrating the production of this PHA class from unrelated and less costly carbon sources as a product of genetically engineered bacterial strains [22, 26].

Over time, the biosynthesis pathways have been increasingly understood and many efforts have been devoted to improve the PHA production efficiency. Since the PHA biosynthesis is competing with other metabolic intermediates, the genetic engineering has acted to remove or weaken competing pathways and so channeling microbial resources to the PHA biosynthesis. The growth pattern and the cell size are also a focus for a rapid proliferation and additional space for polymer production. Clustered regularly interspaced short palindromic repeats interference (CRISPRi) has been used to edit genomes and recently has also been used to control PHA biosynthesis pathway by regulating the metabolic flux, polymer composition and molecular weight. As already well-known the P(3HB) biosynthesis is a multistep enzymatic reaction involving the genes PhaA, PhaB and PhaC. The rational design of ribosomal binding site (RBS) libraries of these three genes has been performed in one-pot reaction by an oligo-linker mediated assembly method (OLMA) resulting in high P(3HB) accumulation, which showed that reprograming the PHA synthesis operons by RBS optimization is another promising alternative to enhance the PHA biosynthesis. The utilization of non-traditional hosts such

as extremophilic bacteria is an additional strategy to improve the PHA biosynthesis. Since these microorganisms grow at specific and extreme conditions, the expression of PHA production genes in extremophiles could prevent microbial contamination by most of microorganisms [28].

4. PHAs Are Promising for Several Applications

The PHA properties such as high biodegradability, biocompatibility, compostability and a variety of monomer composition make the PHAs interesting for a wide range of applications. Despite the packaging sector is still the largest application field for bioplastics which shared almost 40 % of the total bioplastic market in 2016, like other biopolymers the PHAs can be utilized in an infinity of applications including textiles, construction and building, consumer goods, electrics and electronics, agriculture and horticulture films and utensils, automotive industry, besides a variety of medical and pharmaceutical applications [6, 8, 29]. PHAs can be used as matrices for the cultivation of different eukaryotic cells. PHAs have also showed good properties for bone tissue engineering, which can be used added to hydroxyapatite (HA) for hard tissue replacement purposes. Due to low inflammatory response, the PHAs can be utilized as bio-implant patches in the human body. In surgical procedures PHAs can be used for repairing damaged nerves as they are piezoeletric, further, they can also be used for wound dressings and scaffolds. Micro and nanospheres of PHAs are utilized as drug delivery carriers, since these polymers are very biodegradable and biocompatible [30]. Studies have demonstrated that folic acid and Etoposide can be loaded to P(3HB-*co*-3HHx) nanoparticles to selectively target to cancer cells with increased cytotoxicity [31].

As aforementioned there has been reported more than 150 monomers of PHAs. Since the monomer structures are consisted of at least two functional groups including hydroxyl and carboxyl groups, the (R)-hydroxyalkanoic acids can be used as precursors or intermediates for the synthesis of a variety of fine chemicals such as antibiotics, aromatics, pheromones and vitamins, and so opening a wide range of application development. Other interesting application for PHAs is their utilization as a source of biofuels. The esterification of PHAs generates combustion heat comparable to ethanol. As fuel additive the HA methyl esters can be used in ratios of 10-30% for blending with petrochemical fuels like gasoline and diesel. Some properties of 3HB methyl ester has been reported to be similar or better than ethanol as fuel additive which refers to oxygen content, dynamic viscosity, flash point and boiling point. In addition, the metabolic pathways involving P(3HB) synthesis can be adapted to obtain biofuel butanol. Therefore, the application of PHAs as biofuel sources seems to be very promising in a near future [32]. PHAs have been commercially produced since the 1980s and initially they have been used for common applications such as packaging and everyday products

(plastic bags, cosmetic containers, hygiene products, etc.). Nowadays, most large scale PHA producers sells PHAs as raw materials in the form of pellets and powder for extrusion, injection molding, thermoforming and film blowing [33].

Many PHA trademarks have been developed and commercialized. Biopol, a copolymer of P(3HB-*co*-3HV), was firstly produced in 1970s by Imperial Chemical Industries in UK. The patent rights were later obtained by Zeneca, Monsanto and Metabolix. Nodax is a copolymer consisted mainly of 3HB added to medium chain length monomers produced by Procter and Gamble, which is available as foams, fibers, films and many others. Mitsubishi Gas Chemicals has also produced PHAs from methanol under the trade name Biogreen [18]. In 2009, Metabolix and ADM inaugurated the biggest plant for PHA production with a capacity of 50,000 t/a in Iowa, USA. Tianjin Green Bioscience with DSM in China, Meridian in USA, Bio-on in Italy and PHB Industrial in Brazil are examples of PHA facilities with a production capacity of 10,000 t/a. The Newlight Technologies has invested in the PHA production from methane with planed capacity of 43,000 t/a within the next 20 years [33].

Blends of PHAs have been developed to reduce PHA prices and to improve polymer performance. Examples of compounds which were blended with PHAs are poly(latic acid) (PLA), poly(methyl methacrylate) (PMMA), poly(cyclohexyl methacrylate) (PCHMA), poly(ethylene glycol) (PEG), poly(ethylene oxide) (PEO), poly(vinyl alcohol) (PVA), poly(vinyl acetate) (PVAc), poly(caprolactone) (PCL) and poly(butylene succinate) (PBS). Polysaccharides such as cellulose and starch, lignin, chitin and rubbers have also been blended with PHAs [18]. Since the simultaneous synthesis of multiple products is economically desirable for the biotechnology industry, PHAs have been co-produced with a variety of fine chemicals and other value-added metabolites from microbial fermentation. PHAs have been co-produced with α-amylases which permits the utilization of low cost substrates such as starch, wheat and rice bran. Phasins (PhaPs) are predominant proteins in the surface of PHA granules and have been used for bioseparation of recombinant proteins as affinity tags with further separation via centrifugation. The exopolysaccharides (EPS) can be obtained under nutrient imbalance like PHAs, with the first one being produced extracellularly as energy source or protective structures under adverse growth conditions [34]. Both PHAs and EPS production have been reported in *Anabaena cylindrica* [35], *Azotobacter beijerinckii* [36] and *Sinorhizobium meliloti* [37].

The simultaneous production of mcl-PHAs and biosurfactants were reported in *P. aeruginosa* strains utilizing waste cooking oil, glycerol and cassava waste water [38]. Associating PHAs with biofuels is an additional interesting alternative and the co-production of PHAs and methanol has been observed in methanotrophic bacteria [34]. Carlson and Srienc reported the P(3HB) and ethanol production under anaerobic conditions by a recombinant strain of *Saccharomyces cerevisiae* [39] whereas the simultaneous production of PHAs and hydrogen has been observed in the non-purple

sulfur bacteria *Rhodospirillum rubrum, Rhodobacter sphaeroides, Rhodopseudomonas palustris and Thiocapsa roseopersicina* [40]. Organic acids such as succinate has considerable value for the biotechnology industry. Succinate has been simultaneously produced with PHAs by metabolic engineered *E. coli* from glucose, glycerol and fatty acids [34, 41]. As can been seen PHAs are very versatile for the biopolymer industry and many efforts have been devoted to the development of new metabolic routes in order to expand their range of applications. Below, in the next section, it can be seen that the utilization of industrial by-products and wastes is a sustainable alternative to support the PHA production, which can not only improve the economical feasibility of a scaling-up PHA production, but it also corroborates increasingly for a renewable industrial process with environmental responsibility.

5. SUSTAINABLE FEEDSTOCKS FOR PHA PRODUCTION

A sustainable production of PHAs is multi-facetted and needs to attend a process chain involving both environmental and economical feasibility in an industrial scale [42]. The raw materials for microbial cultivations can account up to 50% of the total PHA production cost from which the carbon source reaches up to 80% of the expenses ascribed to raw materials [43]. Accordingly, various scientists have been investigated carbon-rich substrates including industrial by-products and waste materials in order to support the PHA production, not only economically but also environmentally. These inexpensive feedstocks should be available in sufficient amounts and constant quality during the year, maintaining their chemical composition and exhibiting resistance to storage [42]. The sustainability of PHA production has been assessed using many tools such as Life Cycle Analysis (LCA), Carbon Footprint, Carbon Efficiency, Sustainable Process Index, Health and Safety Score Cards and Biomass Utilization Efficiency (BUE). The choice of feedstock is essential to lower the environmental impact of PHA production and even make the material a net carbon sink [33].

A production set based in a biorefinery concept for biofuels and bioplastics partially or totally independent of petroleum-based compounds has been a focus of many authors and research groups [44-46]. Therefore, the utilization of common substrates and by-products from biofuel plants is an interesting alternative to support both industries. The biofuels can be classified into three classes according to their feedstocks. First generation biofuels are produced from edible sources such as grains, oil seeds, sugar and starch crops. They are the less sustainable feedstocks since they compete directly with human food nutrition. Bioethanol from sugarcane or corn crops and biodiesel from fats and oils are examples of first generation biofuels. The second generation biofuels are produced from lignocellulosic materials such as the remaining sugarcane bagasse and cereal straw and short-rotation crops like non-edible grasses. A third class has been created for algal

feedstocks which can not only prevent the utilization of edible sources but it also can spare the agricultural lands [47, 48]. Many of these biofuel feedstocks are also a source for PHA production, including the by-products and residues related to such industries.

Despite advanced biofuels have been developed from chemical synthesis such as pyrolysis and Fischer-Tropsch synthesis, these liquid and gaseous alternative biofuels have been faced many technological challenges [49]. Nowadays, the first generation biofuels bioethanol and biodiesel are still leaders to attend the commercial demand. While biodiesel industry is projected to increasingly grow in the next years and it is predominant in EU countries, the bioethanol industry is well-established comprising the majority of biofuel market with USA and Brazil as the main producers. Corn and sugarcane are the main raw materials for bioethanol production in USA and Brazil, respectively [50]. Besides sugar and ethanol the sugarcane biorefineries generate about 280 kg of sugarcane bagasse and 60 kg of sugarcane molasses by each ton of processed sugarcane [51]. Molasses is a high sugar content source which can be converted to acetyl-CoA and after to PHAs. P(3HB) has been obtained from sugarcane molasses by *Azotobacter vinelandii* [52] and *Bacillus megaterium* [53] and from beet molasses by *Bacillus cereus* [54]. mcl-PHAs can also be synthesized by *Pseudomonas corrugata* utilizing soybean molasses as carbon source [55].

While sugarcane is a source of sugars for bioethanol in Brazil, corn starch is a rich-glucose source which can be obtained from various plants and has been the main raw material for bioethanol production in USA [56]. The PHA production has been reported utilizing starch from various vegetable sources such as corn and rice [57], cassava [58] and potato [59]. Starch is not only a source of glucose for the microbial conversion of biotechnological metabolites but it is also a raw material for thermoplastic starches (TPS) and blending bioplastics [1], offering multiple applications. Food crops, forest and wood residues are also sources of lignocellulosic materials, which are generated in large amounts and they are consisted of cellulose, hemicellulose and lignin. The hydrolysis of these compounds produce sugars such as glucose, xylose and arabinose that can be used for microbial fermentation [60]. These carbon sources are also substrates for PHA production. *Halomonas boliviensis* is a P(3HB)-producing bacteria which is able to utilize the sugars from the enzymatic hydrolysis of wheat bran for polymer synthesis [61]. Lopes et al. have been reported the P(3HB) and P(3HB-*co*-3HV) production by *Burkholderia* sp. from sugarcane bagasse hydrolysate and levulinic acid [62]. Authors from the same group have previously tested different *Burkholderia* strains for P(3HB) production using glucose, xylose and sugarcane bagasse hydrolysate reaching a polymer productivity of 0.47 g/L.h from glucose plus xylose [63].

Vegetable oils from soybean, rapeseed and oil palm have been feedstocks for the biodiesel industry and have been also sources for PHAs [12, 64]. *Aeromonas caviae* is a well-known example of PHA-producing bacteria which is able to synthesize P(3HB-*co*-3HHx) from vegetable oil [65]. The P(3HB-*co*-3HV-*co*-3HHx) production has also been

reported from palm kernel oil and 3HV precursors, sodium propionate and valerate, by a recombinant strain of *C. necator* [66]. *Pseudomonas* strains are also well-known microorganisms which synthesize mcl-PHAs from oleaginous compounds. Four *P. aeruginosa* strains have been tested for mcl-PHA production from waste cooking oil besides cassava waste water and glycerol [38]. This latter, is the main by-product of biodiesel industry which is generated after the transesterification reaction of oils and fats with alcohol for alkyl ester production (biodiesel). The remaining crude glycerol generated from this process accounts about 10% (w/w) of the total reaction. The directly utilization of crude glycerol for the conversion of industrial value-added products is imperative to economically support the biodiesel industry [67]. PHA production from crude glycerol has been one of the possible solutions to aggregate value to both industries. The PHA production utilizing crude glycerol has been reported from classical PHA-producing strains such as *C. necator* [15] to new isolated and until recently unknown PHA producers such as *Pandoraea* sp. [44].

Methylocystis paravus, *Methylosinus sporium*, and *Methylocella tundra* are examples among more than 300 bacterial strains which are able to synthesize P(3HB) from methane, the main component of biogas [68]. The PHA production from activated sludge is an additional alternative to municipal waste management besides the biogas production from anaerobic digestion [69]. Other promising gaseous biofuel is the synthesis gas or just syngas from pyrolysis of biomass sources. The purple non-sulful bacterium *Rhodospirillum rubrum* has been a model organism for the PHA production from syngas [70]. Among recent upgrades of research and development on biofuels, microalgae have been revealed as a future solution for biofuel feedstocks, which can not only provide renewable and non-edible carbon sources, but since they are aquatic microorganisms they could also contribute to the preservation of agricultural lands [48]. Genetically engineered cyanobacteria have been used for P(3HB) production. Cyanobacteria are able to grow in different environments and so they have been considered as promising organisms for PHA synthesis [71].

Many other industrial by-products have been utilized for PHA production [72]. Cheese whey whose the main component is lactose has been a source for P(3HB) synthesis by recombinant *E. coli* [73], *P. hydrogenovora* [74], *Thermus thermophilus* [75] *Methylobacterium* sp. [76] and *Hydrogenophaga pseudoflava* [77]. Many of PHA-producing strains are unable to convert lactose directly into PHAs due to missing or completely lacking β-galactosidase activity, most of lactose sources are subjected to hydrolysis methods to generate the monomers glucose and galactose. As a result of intense R&D activities many gram-negative and gram-positive eubacteria and extremophile archaea have been investigated regarding their ability to synthesize PHAs from an infinity of carbon sources. The cyanobacterial phototrophic conversion of CO_2 towards biomass and PHAs follows a future trend for bioplastic production, which

combines reduction of CO_2 levels from industrial effluent with the generation of biotechnological products [42].

6. Challenges and Future Prospects: A Conclusion

The main challenge faced by PHA industry is the same of bioplastic industry: cost effectiveness. Some bioplastics have lower material performance, especially PHAs which present a still complicated production process with low efficiency, and many times they are utilized for blending with fossil-based polymers, which makes creating high-performance bioplastics at a competitive price a big challenge [78, 79]. In 2004, the price of commercial PHAs was 15-17 times higher than petrochemical polymers and 4-6 times higher than PLA. In 2009, the PHA costs were reduced to approximately US$ 5/kg which was three times higher than PP prices [33]. Indeed the PHAs have encountered many barriers to conquer a well-established market among commodity plastics. Particular big challenges related to PHA production are the costs with raw materials. Glucose has been the main carbon source for microbial cultivations whose prices has continuously increased [78]. Since PHAs are intracellular metabolites, the downstream process to recovery and purify the produced biopolymers contributes significantly to the overall PHA manufacturing costs [80]. Further, the PHA structures and properties are not as consistent as those observed for petroleum-based plastics, for example, their slow crystallization process is a drawback compared to common plastics [78].

The PHA industry has yet to develop additional high value applications [78]. The PHA market is still limited, despite their potential to substitute 33% of conventional polymers [33, 81]. The oscillating oil prices and the existing infrastructure make yet the petrochemical polymers the best choice for plastic market [1]. Consequently, the bioplastic market is dominated by “drop-in” plastics, which are the renewable alternative for fossil-based plastics such as polyethylene (bio-PE), polypropylene (bio-PP), or polyethylene terephthalate (bio-PET). They are identical to their petrochemical counterparts and do not demand infrastructure adaptation. However, these “drop-in” solutions are also not biodegradable as well as the conventional plastics. The bio-based polyurethane (PUR) and “drop-in” PET are market leaders of the bioplastic industry [6, 78]. In 2016, the bioplastic industry income was about US$ 15 billion worldwide compared to US$ 13 billion in 2014 [10]. The production capacity of bioplastics is forecasted to increase from 4.2 million tons in 2016 to 6.1 million tons by 2021 [8]. From 2011 to 2013, the bioplastic share has continuously increased from 1.4% to 2% of world’s total polymer capacity. In fact, the subsequent years have been marked by stagnated values with the production capacity growth dropping from 10% to 4% per year from 2015 onwards [10, 82].

In front of lower oil prices, unfavorable political support and slower growth rate of capacity utilization, some bioplastics such as the high performance PA, the "drop-in" PET and the biodegradable PLA and PHAs are exceptions which have shown fast increase rates of their production capacities. Lowering the costs with carbon sources for bacterial cultivations, improving polymer productivity and extraction methods are strengths which drives the PHA producers in a optimistic way. Several companies have invested in PHAs and among bioplastics the most dynamic development is expected for the PHA industry, whose production capacity is foreseen to grow almost three times by 2021 [10] (Figure 5). The global awareness regarding the environment concerns with the side effects of hundred years of petroleum exploitation makes the market of renewable and sustainable products an increasing global trend [1, 83]. As mentioned above, PHAs have yet faced many technological and economical challenges. On the other hand, the scientists have focused their efforts to overcome such obstacles and they are succeeding, which makes PHAs a promising bioplastic alternative for a near future.

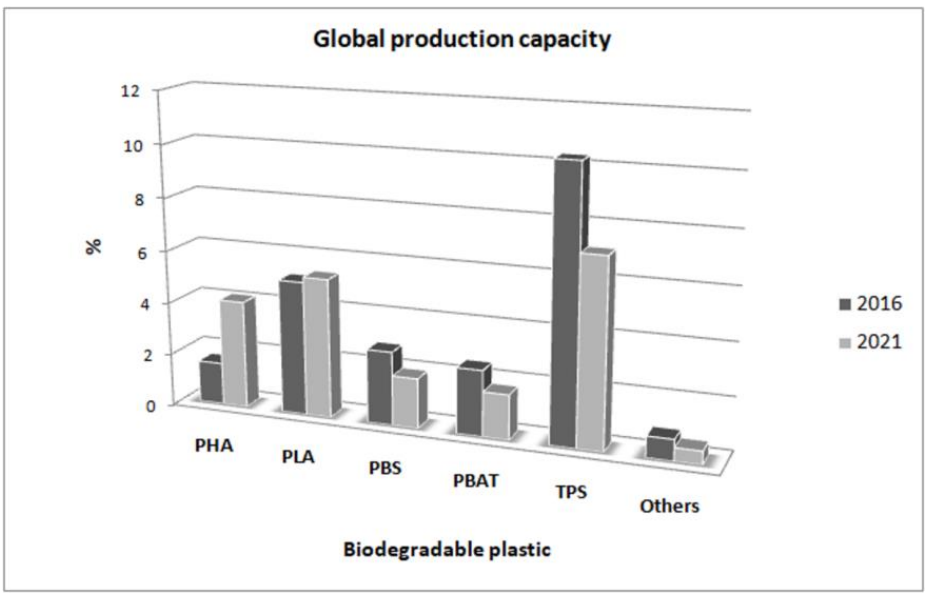

Figure 5. The global production capacity of biodegradable plastics observed in 2016 and expected by 2021 [10].

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Chapter 4

POLYHYDROXYALKANOATES: CHEMICAL STRUCTURE

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ABSTRACT

One of the well-known biopolymer groups generated by direct biosynthesis from renewable resources is the family of Polyhydroxyalkanoates (PHAs). It is a representative category of structurally diverse intracellular biopolyesters accumulated by many bacteria as carbon and energy storage granules. PHA molecules are typically constituted by numerous (R)-hydroxy-fatty acid monomer units. Each monomer unit bears a side chain R group, which is usually either saturated or unsaturated alkyl groups, branched alkyl groups, and substituted alkyl groups, although these forms are less common. PHAs are homo-, co- and terpolymers, generally classified into short-chain-length PHAs (*scl*-PHAs) and medium-chain-length PHAs (*mcl*-PHAs) by the different number of carbons in their repeating units. The main polymer of the PHA family is the homopolymer Polyhydroxybutyrate P(3HB). The number of differences in PHA properties profile depends on the: i) variety of monomers, ii) constitutional isomerism, iii) wide range of molecular weights, and iv) physical and/or chemical modifications of their microstructures. Recent research on structural variations of PHAs has been directed towards the design, biosynthesis, and properties of biodegradable and biocompatible materials, which can be used for bioengineering of new optical and other smart chiral intermediates. Moreover, PHAs are exploited in a series of applications in the packaging and food industry, agriculture, medicine, pharmacy, and also as raw materials for

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synthetic chemistry, representing an interesting source for smaller molecules or chemicals. Therefore, due to their commercial interests, PHAs are a promising group of materials for future study related to synthetic mechanisms, monomer diversity, physiological roles, and controllable production.

Keywords: polyhydroxyalkanoates, biopolymers, biopolyesters, chemical structure

1. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a family of structurally complex macromolecular biopolymers synthesized under restricted nutrients growth conditions, by various Gram-positive and Gram-negative bacteria, and accumulated as intracellular carbon and energy storage granules to amounts as high as 90% of the cell dry weight [1, 2]. PHAs are mainly produced from renewable resources by fermentation, and are classified as environmentally friendly materials. Their biodegradable and biocompatible properties render them suitable for short term packaging, and for biomedical applications such as drug encapsulation and tissue engineering. PHA degradation process can be either abiotic occurring by a simple hydrolysis of the ester bond without enzymatic catalysis or biotic by enzymatic degradation until final mineralization [3].

It has been 91 years since the discovery of the homopolymer poly(3-hydroxybutyric acid), P(3HB), as an energy and carbon storage macromolecule from the bacterium *Bacillus megaterium*. Its monomer unit, D(-)-3-hydroxybutyric acid (3HB), constitutes the most well-known component of PHAs [4]. Later in the early 80's, 3-hydroxyvaleric acid (3HV), 3-hydroxyhexanoic acid (3HHx) and 3-hydroxyoctanoic acid (3HO) were discovered incidentally, as monomeric components of PHAs accumulated by axenic cultures of *Bacillus sp.* [5], *Alcaligenes eutrophus* [6], and *Pseudomonas oleovorans* [7], respectively. An earlier overview based on the diversity of microbial PHAs has been contacted, and the occurrence of various hydroxy-alkanoic acids (HAAs) as the main PHA components has already been extensively recorded [8].

Until today, a wide range of PHA homopolymers and copolymers have been produced at the laboratory scale. P(3HB), the principal homopolymer of PHAs, constitutes the basic synthon for the production of different copolyesters such as poly(hydroxybutyrate-*co*-hydroxyalkanoates) (PHB-*co*-HAs), poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHB-*co*-HV), poly(hydroxybutyrate-*co*-hydroxyhexanoate) (PHB-*co*-HHx), poly(hydroxybutyrate-*co*-hydroxyoctanoate) (PHB-*co*-HO) or poly (hydroxybutyrate-*co*-hydroxyoctadecanoate) (PHB-*co*-HOd), and others [9, 10].

Nowadays, PHAs have become the major source of thermoplastics with over 125 different types prepared from various hydroxyl-alkanoate (HA) monomers. The various differences in PHA properties depend on: a) the co-monomer structure, b) the length of

pendant groups, c) the length of carbon chain in the repeating unit of the polymer backbone, and d) the mol% co-monomer content in the polymeric chain [3]. The tailoring of these properties affects the bioengineering of the bacterial genome towards the production of different types of PHAs with various industrial applications, with regard to the chemical modification processes, degradation characteristics, and material properties.

2. Basic Chemical Structures of PHAs and Classification

The general chemical structure of PHAs is shown in Figure 1, where **R** group and ***m***, ***n*** numbers are variable generating the vast family of PHAs.

The majority of the identified PHAs are primarily linear, head-to-tail polyesters with 3-hydroxy fatty acid monomers as basic structural units [2]. During the synthetic procedure of these polymers, the carboxyl group of one monomer unit forms an ester bond with the hydroxyl group of the adjacent monomer [2]. In general, apart from some special types of PHA structures where no chirality is observed, the hydroxyl-substituted carbon atom is of the R configuration [2] (Figure 1 and Table 1). However, fatty acids with the hydroxyl group at the γ, δ, or ε position have also been incorporated [2]. C-3 or β position can be obtained by an alkyl group which varies from methyl to tri-decyl moiety [2].

PHAs are generally classified by the variable number of carbons in their repeating units into short-chain-length PHAs (*scl*-PHAs) with 4 or 5 carbons, medium-chain-length PHAs (*mcl*-PHAs) with 6-14 carbons, and long-chain-length PHAs (*lcl*-PHAs) with 15 or more carbons. Additionally, PHAs are classified as homo-polymers (Figure 1 and Table 1) or co-polymers according to the similarity or diversity of their composition in monomer units, which depends entirely on the specificity of PHA synthases enzymes [11].

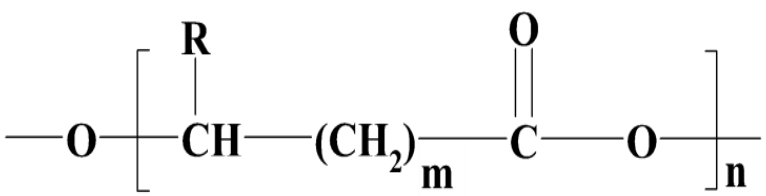


Figure 1. General chemical structure of PHAs where ***m*** = 1, 2, 3. ***m*** = 1 is the most common. ***n*** can range from 100 to several thousands. **R** is variable. When ***m*** = l and **R** = CH_3 the monomer structure corresponds to 3-hydroxybutyrate (3-HB). When ***m*** = 1 and **R** = C_3H_7 the structure corresponds to 3-hydroxyhexanoate (3-HHx) monomer.

2.1. PHA Homopolymers

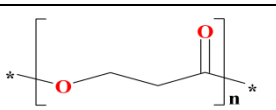
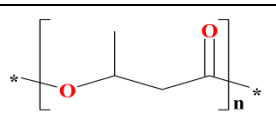
P(3HB) is the most extensively studied and commercially triggering homopolymer of PHAs. P(3HB) is easily accumulated by bacteria and this ability is often used as a taxonomic characteristic. P(3HB) is a biopolymer with quite limited applications due to its high crystallinity. However, incorporation of co-monomers can modify the P(3HB) structural characteristics enhancing the flexibility of the polymer chain and thereby reducing its crystalline character.

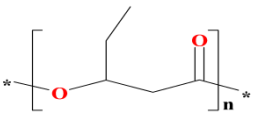
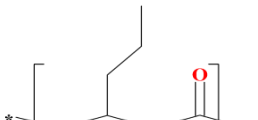
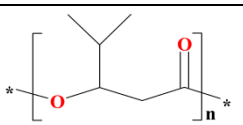
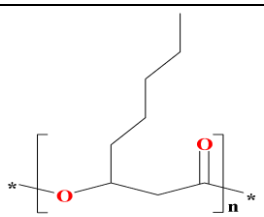
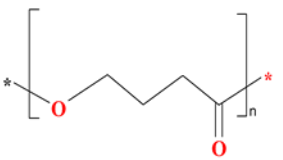
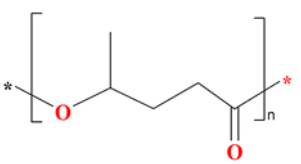
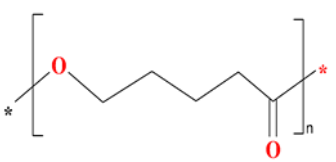
Recent advances in the molecular biology and biochemistry of PHA biosynthetic procedures have resulted in the development of the cloning of PHA biosynthetic genes from a variety of bacteria [11, 12]. These facts have evoked new research directives for the growth of recombinant bacteria capable of PHA production directed by heterologous genes [13]. Research results have proved that recombinant *Escherichia coli* harboring PHA biosynthetic genes from *Ralstonia eutropha* yield P(3HB) homopolymer of molecular weight values between $3x10^6$ - $1.1x10^7$ under specific fermentation conditions [14].

P(3HB) stretched films from the high molecular weight P(3HB), with improved mechanical properties compared to the unstretched film, have been also successfully prepared [14]. Additionally, it has been observed that annealing treatment of the stretched film improves its mechanical properties. Collectively, genetic engineering techniques can upgrade P(3HB) homopolymer physical properties rendering it a prospective candidate for future commercial utilization [15].

Although a plethora of PHAs have been produced by various biological sources, only a few biosynthetic homopolyesters such as 3HB, 3HV, 3-hydroxy-5-phenyl-valeric, 3HHx, 3-hydroxyheptanoate (HHp), 3HO, 3-hydroxynonanoic (3HN) and 4HB are available [8], yet not suitable for large scale production apart from P(HV) and P(4HB). Other representative homo-polymeric PHA structures are shown in Table 1.

Table 1. Indicative homopolymer PHA structures

Chemical name	*m* value	R group
 Poly(3-hydroxypropionate) PHP	1	Hydrogen
 Poly(3-hydroxybutyrate) PHB (P3HB)	1	Methyl

Chemical name	*m* value	R group
 Poly(3-hydroxyvalerate) PHV (P3HV)	1	Ethyl
 Poly(3-hydroxyhexanoate) PHHx (P3HHx)	1	Propyl
 Poly(3-hydroxymethyl-valerate) PHMV (P3HMV)	1	Isopropyl
 Poly(3-hydroxyoctanoate) PHO (P3HO)	1	Pentyl
 Poly(4-hydroxybutyrate) PHB (P4HB)	2	Hydrogen
 Poly(4-hydroxyvalerate) PHV (P4HV)	2	Methyl
 Poly(5-hydroxyvalerate) PHV (P5HV)	3	Hydrogen

2.2. Production of PHA Heteropolymers (or Copolymers)

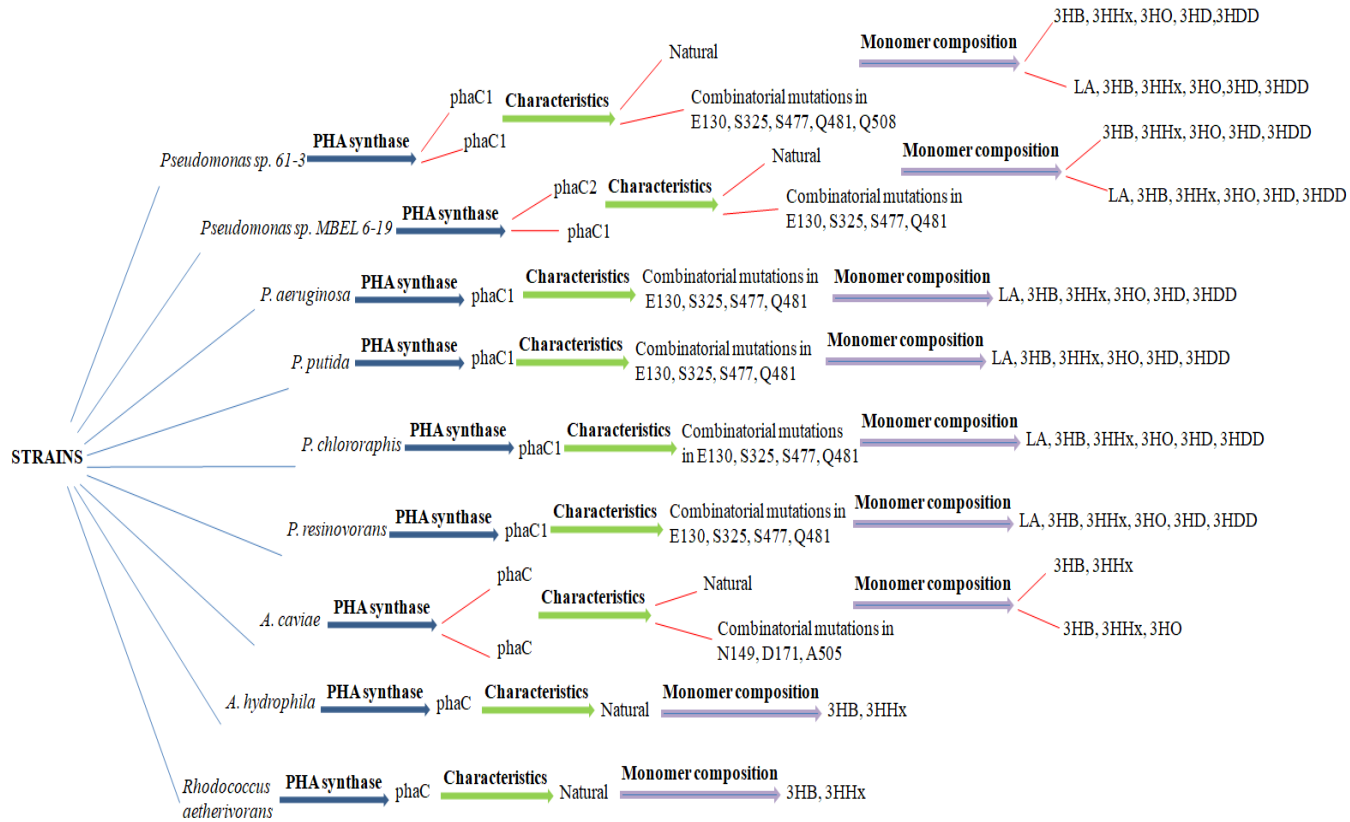
Direct production of PHAs through the process of fermentation offers a large variety of structures with more than 150 diverse HAs and mercaptoalkanoic acid as basic constituents [16]. The affinity with the microorganism host and the growth substrate, the specificity of the corresponding PHA synthase of each bacterial strain employed, and the carbon sources fed during the fermentation procedure strongly affect the length of the polymer chain of the produced PHA, functioning as guides for the synthesis of homo-, copolymers or blends [17]. Collectively, both the factors of PHA synthase specificity and PHA structure correlations with carbon substrates and host microorganisms are extensively discussed below.

2.2.1. Specificity of PHA Synthases

Generally, PHA synthases are the most crucially-operating PHA biosynthetic enzymes, utilizing various HA-CoAs as substrates and incorporating the suitable monomer units, undergoing polymerization, to the PHA chain. They are categorized into four classes (class I, II, III, and IV) according to their subunit compositions and substrate specificities [9]. PHA synthases of the first two classes are constituted by one subunit PhaC enzyme. *Alcaligenes latus* and *Ralstonia eutropha* are representative species of PHA synthases Class I with molecular weights ranging from 61 to 68 kDa. The *in vivo* substrate specificity of PHA synthases Class I defines the employment of coenzyme A thioesters for various 3-*scl*-HAs consisting of 3-5 carbon atoms, whereas PHA synthases Class II originated mainly from *Pseudomonads*, utilize 3-*mcl*-HAs. [18, 19].

Representative examples of PHA synthases Class II such as *Pseudomonas sp.* 61-3 [20], and *Pseudomonas sp.* 6-19 [21] employ both *mcl*- and *scl*- monomers with negligible activity towards *scl*- monomers. The subunits PhaC and PhaE are the main constituents of PHA synthases Class III and they possess marginal sequence homology to PHA synthases class I and II. PHA synthases Class III present high specificity for 3-*scl*-HAs, with a few exceptions of 3-*mcl*-HA specificity after expression in some pseudomonas [22]. Finally, class IV PHA synthases are constituted by two different subunits, PhaC and PhaR, which are mostly found in *Bacillus* strains producing intracellular granules of P(3HB) [23, 24].

A large variety of HA-CoAs function as substrates of PHA synthases and after polymerization they form PHA polymer granules inside the cellular milieu [9, 25, 26, 27]. Indicative PHA synthases for the synthesis of PHAs with both *scl*- and *mcl*-monomers are presented in detail in Scheme 1 [9].



Scheme 1. Indicative PHA synthases for the synthesis of PHAs with both *scl*- and *mcl*-monomers [9].

PHA synthases class I, III, and IV are able to utilize as substrates the 3HV-CoA, 3HB-CoA, and 4HB-CoA *scl*-monomers. This incorporation to the P(3HB) polymer main body has been achieved through the employment of natural PHA synthases derived from *Alcaligenes latus*, *Ralstonia eutropha*, and *Allochromatium vinosum*. Due to the high substrate specificity of natural PHA synthases, the incorporation of both *scl*- and *mcl*-monomers into the P(3HB) backbone is not sufficient, leading to the development of PHA synthases suitable for the generation of P(3HB) copolymers with a small amount of *mcl*-monomers [9].

The PHA synthase purified from the thermophilic bacterium *Thermus thermophilus*, grown in sodium gluconate, is constituted by one subunit of 55 kDa, and probably belongs to a new class of PHA synthases since it produces *lcl*-PHAs of unusual composition containing mainly 3-hydroxydecanoate (3HD) with a molar fraction of 64% [28].

2.2.2. PHA Structure Correlations with Carbon Substrates and Host Microorganisms

Substrates provided in the culture as carbon sources suitable for PHA production, are transported into the cell either by a simple diffusion or by specific membrane-located transporters. Then, they are introduced into a cycle of catabolic and/or anabolic reactions, and they are converted into hydroxyl acyl coenzyme A thioesters, which constitute the substrates of PHA synthases. The degree of PHA synthases specificity towards these hydroxyl acyl coenzyme A thioesters reflects in the composition of PHAs, resulting in either homo- or hetero-polyesters.

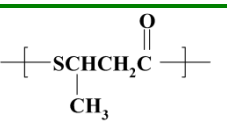
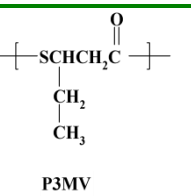
One of the most efficient and exclusive *scl*-PHA-producing microorganism is *Ralstonia eutropha*, functioning as a recombinant host strain and synthesizing PHAs from a variety of renewable low-cost carbon sources, plant oils and sugars [3].

The bacterial strains *Aeromonas hydrophila* 4AK4, *Escherichia coli* S17-1, or *Pseudomonas putida* KT24424 produce 4-Hydroxybutyrate (4HB) through the transformation of 1,4-butanediol (1,4-BD) to 4HB, by harboring 1,3- propanediol dehydrogenase gene dhaT and aldehyde dehydrogenase gene aldD from *P. putida* KT2442. The production of homopolymer poly-4-hydroxybutyrate P(4HB) and copolymers poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] was achieved through the utilization of fermentation broth with 4HB. The most effective 4HB source was the recombinant *Aeromonas hydrophila* 4AK4 bacterial strain harboring plasmid *pZL-dhaT-aldD* containing *dhaT* and *aldD I II* [29].

An unusual novel class of bio-copolymers synthesized by PHA-producing bacteria is the polythioesters (PTE), containing 3-mercaptopropionate (3MP), 3-mercaptobutyrate (3MB), and 3-mercaptovalerate (3MV) incorporated as monomeric units in the polymeric chain of PHAs, apart from the 3HB. Moreover, polythioesters (PTE) homopolymers such as P3MP, P3MB, and P3MV were reported to be produced by a recombinant strain of

Escherichia coli able to express an unnatural PTE biosynthetic pathway [30]. The PTE structures as well as their T*m* values are presented in Table 2.

Table 2. Chemical structures and T*m* values of poly(3-mercaptoalkanoate)s (PTE homopolymers) P3MP, P3MB and P3MV [48]

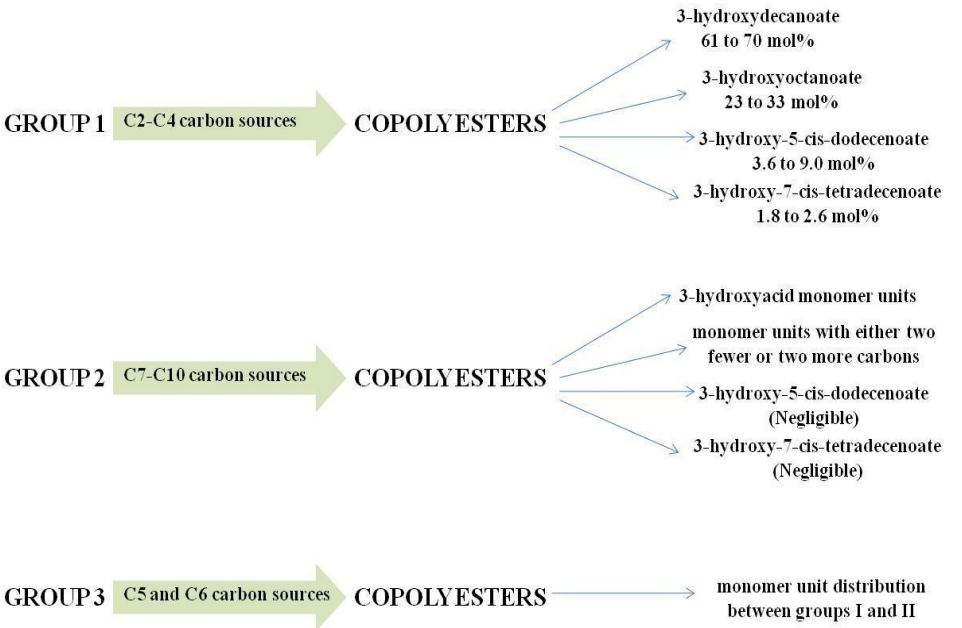
PTE structures	 P3MP	 P3MB	 P3MV
T*m* values	170°C	100°C	84°C

Utilization of specific carbon sources in PHA production offers a great variety of functionalities in the side groups. The modified *mcl*-PHAs can be obtained by *de novo* fatty-acid biosynthesis through transformation of glycerol or sugars into unsaturated polyesters or by feeding substrates with structural similarities after processing via the beta-oxidation pathway. The produced materials possess enhanced thermal and mechanical properties providing a diversity of applications. In that respect, PHAs bearing side chains with terminal double bonds, ensure production rates of high yield [31], susceptibility to chemical alterations, while the corresponding alkylic substrates bearing double bonds are inexpensive with a nontoxic profile [32, 33]. Moreover, *scl*- and *mcl*-PHAs bearing double bonds may also be a product of recombinant *Methylobacterium extorquens* strains after feeding with unsaturated fatty acids [34]. These kinds of methylotrophic microorganisms offer the advantage of using methanol as an inexpensive and abundant, non-food substrate, in contrast to poly(3-hydroxy-10-undecenoate) P(HU), or other unsaturated polyesters that constitute the cultivation products of various types of pseudomonas over expensive or food-substrates such as vegetable or animal edible oils, 1-alkenes and 10-undecenoic acid or oily acids [35, 36, 37].

Poly(3-hydroxy-4-pentenoic acid) P(HPE), is and indicative unsaturated *scl*-PHA. In this case, PHA accumulation is induced by the growth of *Burkholderia sp.* on a sucrose-containing mineral salts medium, with limited concentration of phosphates. These conditions enhance the bacterial synthesis of polyesters with 3HB and 3-hydroxy-4-pentenoic acid (3HPE). The fractionation of the solvent used for the purification of the polyester indicates the existence of both P(3HB) and P(3HPE) homopolymers, instead of a co-polyester with random monomer distribution [38]. The properties of crystallinity and melting temperature of the P(3HPE) homopolyester are lower than these of P(3HB) and P(HV) [39]. The growth of *Rhodospirillum rubrum* on 4-pentenoic acid and on a 4-pentenoic acid/n-pentanoic acid mixture 1:1 (mol:mol) results in the incorporation of 3HPE in a HB-*co*-HV-*co*-HPE terpolyester [40, 41].

Literature reports on 42 different carbon sources for polyester synthesis by *Pseudomonas citronellolis* (ATCC 13674), including linear C2-C10 monocarboxylic and C3-C10 dicarboxylic acids, a,ϖ-diols, saccharides, hydrocarbons, and 3-methyl-branched substrates such as 3-methyl-n-valerate, 3,7-dimethyl-6-octen-1-ol (citronellol), 3-methyladipate, and 3-methyl-1-butanol [42] showed that the produced polyesters can be classified into three groups as presented in Scheme 2. Indicatively, the synthesis of the novel copolyester, poly(3-hydroxy-7-methyl-6-octenoate-co-3-hydroxy-5-methyl-hexanoate), was achieved after growth on citronellol [43].

Pseudomonas oleovorans bacterial strain has been used for the production of PHAs bearing phenoxy groups in the side. The carbon source used for the bacterial culture was phenoxyundecanoic acid and the obtained polyester was poly(3-hydroxy-5-phenoxypentanoate-co-3-hydroxy-9-phenoxynonanoate) copolymer of 5:6:1 molar ratio [45].



Scheme 2. Carbon substrate-dependent classification of PHA copolyesters [44].

Cultivation of *Pseudomonas oleovorans* in the presence of variable molar ratios of two substrates 5-(2′, 4′-dinitrophenyl)valeric acid (DNPVA) and nonanoic acid, results in the production of polymers with incorporated 4′-nitro- or 2′, 4′-dinitrophenyl group substitution and at a percentage ranging from 1.2-6.9% of repeating units [46]. An indicative example of a methyl-branched PHA, grown on mixtures of n-octanoate with methyloctanoates is poly(3-hydroxy-6-methylnonanoate), P(H6MN) [47]. Respectively, cultivation of *Pseudomonas cichorii* YN2 on carbon substrates of C7–C12 1-alkenes resulted in PHAs containing repeating units with terminal epoxide groups [48].

An indicative example of a PHA containing an epoxide group is presented in Figure 2.

Copolymers of P(3HB) containing 3HV or 4HB monomers constitute a class of PHAs characterized as short-side-chain PHAs (*ssc*-PHAs), and can be formed by co-feeding of substrates. Additionally, PHAs composed of C6 to C16 3-hydroxy fatty acids are characterized as medium-side-chain PHAs (*msc*-PHAs). These types of PHAs are synthesized from fatty acids or other aliphatic carbon sources, and their compositions depend on the growth substrate used [49, 50, 51]. Although *msc*-PHAs can also be synthesized from carbohydrates, their composition is not related to the carbon source [52, 53, 54]. *Ssc*-PHAs with 3HB as the primary unit or *msc*-PHAs with 3HO and 3HD as the major monomers are the main synthetic products of the majority of microbes [55, 56, 57, 58].

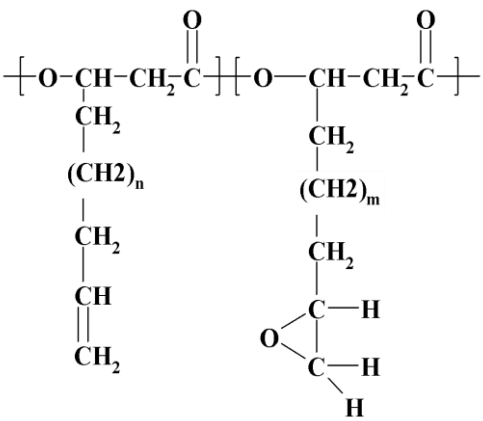


Figure 2. PHA with an epoxide group [44].

PHAs produced from the thermophilic bacterium *Thermus thermophilus*, using sodium gluconate as carbon source, are *mcl*-PHAs of unusual composition containing mainly 3-hydroxydecanoate (3HD) with a molar fraction of 64%, also including 3HO, 3HV and 3HB. The heteropolyester derived from octanoate-grown cells consisted of 24.5 mol% 3HB, 5.4 mol% 3HO, 12.3 mol% 3-hydroxynonanoate (3HN), 14.6 mol% 3HD, 35.4 mol% 3-hydroxyundecanoate (3HUD) and 7.8 mol% 3-hydroxydodecanoate (3HDD) [**28**]. A copolymer consisting of *scl* 3-HV (38 mol %) and *mcl* 3-HHp, 3-HN, and 3-HUD (35.42 mol%) was also produced when this bacterium was cultivated in 24% v/v whey medium [59].

A recent overview records the various substrates, microorganisms, and recent advances on the improvement of the performance of PHAs and their copolymers in combination with novel approaches towards their inexpensive production [60].

A successful synthetic effort led to the production of a wide range of *mcl*-PHA homopolymers ranging from poly(3-hydroxyheptanoate) P(HHp) to poly(3-

hydroxytetradecanoate), using the *β*-oxidation pathways of engineered *Pseudomonas entomophila* LAC23, grown in the presence of various fatty acids. In addition, the LAC23 strain is also employed to produce random copolymers of 3-hydroxyoctanoate (3HO) and 3-hydroxydodecanoate (3HDD) or 3-hydroxytetradecanoates, and their constitution could be monitored by regulating the proportions of two related fatty acids.

Additionally, the block copolymer P(3HO)-*b*-P(3HDD) is synthesized by the same strain. Interestingly, the even- and odd number *mcl*-PHA homopolymers possess different physical properties. The replacement of the PHA synthase gene in the engineered *Pseudomonas entomophila* by the phaC from *Aeromonas hydrophila* 4AK4, produces the copolymer poly(3-hydroxybutyrate-co-30 mol%-3-hydroxyhexanoate), and therefore *Pseudomonas entomophila* is suggested as the most adequate bacterial strain for the synthesis of the entire spectrum of *mcl*-PHA homopolymers (C7-C14), random- and block copolymers [61].

Recent literature reports imply that the sustainability of the large-scale production of microbial biopolyesters such as PHAs, requires careful consideration of economic, ethical, environmental, and engineering aspects. Novel hetero-, mixo-, and autotrophic PHA production methods, based on various industrial residues from different branches, are also reported. Emphasis is given on the integration of PHA production based on selected raw materials into "the holistic patterns of sustainability". This encloses the selection of novel, potent microbial production strains, non-dangerous, environmentally harmless methodologies for PHA recuperation and recycling of waste streams from the PHA production process [62].

Novel synthetic and biological methods were developed to diversify the PHA structures into homo-, random, and block polymers, with improved properties, in order to confront the plethora of application requirements. Concurrently, different pathways were assembled in order to produce various PHAs by utilizing glucose as a facile carbon source. Finally, *Halomonas* bacteria were reconstructed to generate PHAs with a modified morphology, for inexpensive production, and under non-sterile and uninterrupted conditions. It is expected that the synthetic biology will transform the procedure of PHA production into an industry of bio-materials [63].

The CRISPRi (Clustered regularly interspaced short palindromic repeats interference), is an effective approach for targeted gene inhibition, which may be used for additional metabolic engineering applications in non-model organisms, utilized in the production of PHAs. Indicatively, a non-model microorganism *Halomonas* species TD01, functions as a promising industrial producer of PHAs, and the controllable gene repression system, CRISPRi, was used to regulate its gene expression levels. It was successfully planned to target genes of ftsZ, prpC and gltA, obtaining longer cell sizes, and channeling more substrates to PHBV and PHB synthesis, respectively. CRISPRi was utilized to control expression of gene (prpC), which encodes 2-methylcitrate synthase, targeting the regulation of 3HV monomer proportion, present in P(HBV) copolymers of

3HB and 3HV. Percentages of HV in PHBV copolymers were controllably ranged from less than 1 to 13%. Furthermore, when the gene gltA encoding citrate synthase is repressed, this reflects in channeling more acetyl-CoA from the tricarboxylic acid (TCA) cycle to P(3HB) synthesis [64].

A novel approach towards the improvement of PHA production and diversity constitutes the use of synthetic biology and metabolic engineering. Synthetic biology led to PHAs with controllable composition of random copolymers, homopolymers, and block copolymers. Recent achievements demonstrated the possibility of creating a microbial platform in order to produce not only accidental copolymers with controllable monomers and their proportions, but also homopolymers and block copolymers of specific structure. This purpose was accomplished by engineering the genome of *Pseudomonas putida* or *Pseudomonas entomophiles* by attenuating the biosynthetic pathways of *β*-oxidation and *in situ* fatty acid supplies, targeting the retainment of their original chain length and structures upon their incorporation into the PHA chains. The engineered bacteria allow the functional groups of a fatty acid to be embodied inside the PHA structure, enabling the creation of functional PHAs with various structural diversities [64].

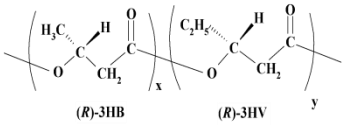
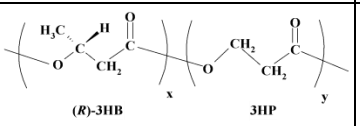
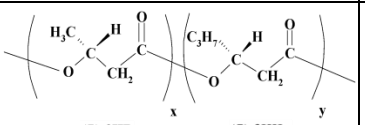
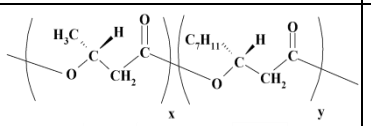
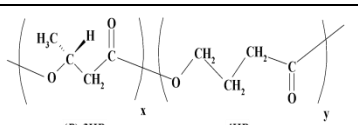
The efficient production of the most active member of PHAs, the poly(3-hydroxypropionate) or P(3HP), was achieved through the utilization of recombinant *Escherichia coli*. Synthetic pathways of P(3HP) and its copolymer P(3HB-*co*-3HP) of 3HB and 3-hydroxypropionate were developed through synthesis from glucose. The successful employment of an advanced CRISPRi for the effective manipulation of multiple genes and observance of the metabolic flow in *Escherichia coli*, led to the production of a variety of P(3HB-*co*-4HB) copolymers. Moreover, the bacterial shapes were efficiently modified, thus increasing the PHA production [65].

Indicative structures of PHA copolymers are presented in Table 3.

2.2.3. PHA Polymer Blends

In order to optimize the P(3HB) physical properties, scientists have developed polymer blends of biodegradable polymers with P(3HB). The polymer blends are considered physical mixtures of two polymers with different structures, either heterogeneous or homogeneous phases, in microscopically amorphous regions at equilibrium. A single phase mixture is regarded miscible. On the contrary, a two-phase mixture is regarded immiscible. The phase structures strongly affect the physical properties of a mixture. Miscible P(3HB) blends have been synthesized with poly(-vinyl alcohol) [66, 67], poly(ethylene oxide) [68, 69, 70, 71], poly(lactide) [72, 73], atactic poly(3-hydroxybutyrate) [74, 75, 76, 77, 78], poly(butylene succinate-*co*-butylene adipate) [79], poly(butylenesuccinate-*co*-e-caprolactone) [79], and poly(ε-caprolactone-*co*-lactide) [80]. Mixtures of P(3HB) with poly(ethylene adipate) [81], poly(β-propiolactone) [81, 82], poly(ε-caprolactone) [83] and poly(butylene adipate) [83] are immiscible.

Table 3. Indicative structures of PHA copolymers [44]

Random copolymer	Carbon substrate	Bacterial strain
 (*R*)-3HB (*R*)-3HV	Propionic acid	*Ralstonia eutropha*
 (*R*)-3HB 3HP	Pentanoic acid and 3-Hydroxy-propionic acid	*Ralstonia eutropha*
 (*R*)-3HB (*R*)-3HHx	1,5-Pentanediol Plant oils	*Alcaligenes latus* *Aeromonas cavie*
 (*R*)-3HB (*R*)-3HD	Sugar 4-Hydroxybutyric acid	*Pseudomonas sp.* *Ralstonia eutropha*
 (*R*)-3HB 4HB	γ-Butyrolactone 1,4-Butanediol 1,6-Hexanediol	*Alcaligenes latus* *Comamonas acidovorans*

2.2.4. Structural Diversities of PHAs

The respective alkyl chain R of PHAs (Figure 1) can be saturated, aromatic, unsaturated, halogenated, epoxidized and branched [47, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94]. Until today, carefully selected unnatural monomers have been incorporated in order to obtain novel polymers with specific properties, such as 4-cyanophenylvalerate [95]. Variability is also observed in the position of the hydroxyl group, with 4-, 5-, and 6-hydroxyl acids being incorporated [96, 97, 98, 99, 100]. The substituent in the side chains can be chemically modified (eg. by cross-linking of unsaturated bonds), offering differentiation in the length and composition and concurrently in the structural and applicational diversity of PHAs [101, 102, 103]. Recent literature reports provide an analytical description of the recent advancements of PHAs in addition with the structure and classification of their copolymers [60].

PHA copolymers are obtained from mixed culture production and can be random in sequence [104]. More specifically, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV), or PHBV, is based on a random arrangement of two monomers with R = methyl and R = ethyl and respectively, poly(3-hydroxybutyrate-*co*-3-hydroxy-

hexanoate) consists of two monomers with R = methyl and R = propyl. For the mixed culture production of PHAs, various types of complex waste feedstocks are used such as paper mill effluents, sugar cane molasses, fermented olive oil mill effluents, fermented municipal sludge, industrial and domestic wastewaters etc. [105]. Genetic and metabolic engineering of the microbial strains enable the regulating procedure of the polymerizing enzyme PHA synthase which is responsible for the successful incorporation of different monomers in the polymer chains [15].

Literature reports indicate that odd-C substrates lead to the synthesis of PHA polymers with odd-C monomers and respectively, even-C substrates lead to the synthesis of PHA polymer chains with even-C monomers. It has also been reported that unsaturated monomers can be successfully incorporated by using a 1-alkene carbon source [106]. Mixed culture production offers a lot of advantages such as cost reduction due to the use of easily available cheap feed stocks, environmentally friendly ways of managing waste, and more favorable life cycle inventories (LCI), i.e., the energy consumption and carbon dioxide emissions from "cradle to reincarnation," for the PHAs random copolymers compared to pure P(3HB). Indicatively, it has also been reported that the values of energy consumption and carbon dioxide production for the PHBHx copolymer are lower compared to the corresponding values for the P(3HB) polymer [107].

3. PROPERTIES OF PHA COPOLYMERS

P(3HB) and its related copolymers represent a family of PHAs with ideal properties, such as stiffness and ultimate tensile strength, necessary for various applications. However, their significantly low flexibility and brittle character render them unsuitable for commercialization. As a result, copolymerization of 3HB with a variety of monomers that ensures improved flexibility, limited breakage and reduced melting point temperature can be a valuable answer to the problem [9, 108]. P(3HB) copolymerization with HV has been reported under specific feedstock and bioprocessing conditions, which affords a P(HBHV) polymer with improved elastomeric properties and higher commercial viability than P(3HB) [109]. In general, although numerous PHA copolymers have been synthesized, very few of them have attracted industrial interest and are commercialized. Improved elasticity and modulus properties of PHA copolymers can be obtained through careful manipulation of melting point and T_g, allowing the use of milder conditions during the processing (e.g., melt temperatures).

Generally PHAs, as linear aliphatic polyesters, possess better resistance against UV radiation than polypropylene [110, 111]. It has been reported that a specific PHA, (PHA-I6001 from Metabolix with a crystallinity of 25%), was blended with a UV sensitive polymer such as PVC, in order to produce a copolymer with less biodegradability and

higher UV resistance. The miscibility of the mixture did not lead to phase separation in the blend, leading to an offset of the PVC UV-induced yellowing, providing color hold during the UV exposure [108].

3.1. Crystallinity of PHAs

PHAs are optically active bio-polyesters with the HA monomers characterized by an R configuration due to the stereo-specificity of the polymerizing enzyme. Although P(3HB) presents high crystallinity outside the cellular milieu, *in vivo* P(3HB) exists as an amorphous molecule [15]. Amorphous PHA molecules were also produced *in vitro* indicating that the crystallization process during the extraction of PHAs is accelerated by the coalescing of the polymer chains. PHA crystallization kinetics after processing is also an important parameter in the determination of the final properties of the materials. More specifically, the crystalline forms and the utilization of nucleating agents for the reduction of the crystalline character of the PHA copolymers influence the manageability of the produced biopolymers [3].

In the past, it was believed that the presence of *in vivo* amorphous PHAs is a result of plasticizers or nucleation inhibitors within the cell inclusion [112]. P(3HB) can be crystallized from dilute solutions or into spherulites after preparation from melt processing resulting in lamellar crystalline structures. A typical crystal of P(3HB) is lath-shaped with 5–10 mm length and a thickness of 4–10 nm [113]. P(3HB) adopts a very stable a-form helix conformation or a less common β-form planar zig-zag conformation. The α-conformation affords lamellar crystals and the β-conformation occupies the amorphous regions between the α-form lamellar crystals [114]. The β-conformation is usually formed by extending the uni-axially oriented films of the P(3HB) further [115].

The *mcl*-PHAs produced from mixed cultures obtain helix conformations forming an orthorhombic lattice structure with two molecules per unit cell. The increase in the length of the side chains in the co-monomers enhances the thickness of the lamellar sheets rendering them more stable and ordered [44]. The mol% concentration of the co-monomer also affects the crystallinity of copolymers. Literature reports on the evaluation of the intermolecular interactions in P(HBV) crystal structures with an HV content less than 30%, indicated that the strength of the H-bonds between the methyl group and the carbonyl group of the HB part was similar to that of P(3HB), and increases with an increase in the HV content [116]. Additionally, the transition from a P(3HB)-type of crystal to a P(HV)-type arises at around 50% HV content. Similar observations have also been reported for other copolymers such as P(3HB-*co*-HP) and P(3HB-*co*-HHx). In the case of the latter, the crystal structure is only slightly affected by the HHx content [51].

The structural properties of a wide range of copolymer compositions of (*R*)-3HB and (*R*)-3HV have been extensively investigated, with P(3HB-*co*-3HV) copolymers possessing the same high degrees of crystallinity (50-70%) [117]. An indicative structural characteristic of P(3HB-*co*-3HV) copolymer is isodimorphism, which is the co-crystallization of the two monomers in either of P(3HB) and P(3HV) crystal lattices, associated with the range of the (*R*)-3HV composition which can vary above or below ~40 mol% [35]. In the case of (*R*)-3HB and (*R*)-3HHx random copolymers with a propyl side chain, the increase of the (*R*)-3HHx unit (0→25 mol%), provoked the decrease of the corresponding X-ray crystallinities (60→18%).

The crystallographic parameters of the P(3HB-*co*-3HHx) copolyesters were insignificantly influenced by the presence of the (*R*)-3HHx units, indicating the exclusion of (*R*)-3HHx from the P(3HB) crystalline phase. Additionally, the increase in the (*R*)-3HHx fractions reduced the crystal growth rates for P(3HB-*co*-3HHx), indicating that the random distribution of the (*R*)-3HHx fractions in the copolymer induced a significant decrease in the deposition rate of the (*R*)-3HB segments during the development of the P(3HB) crystal lattice [118].

Research results on the determination of the structural characteristics for P(3HB-*co*-3HP) copolymers indicated that only one P(3HB) crystalline form was observed for copolymers with 3HP compositions of up to 43 mol%. The presence of 3HP had insignificant impact on the crystallographic parameters, and the increase of the 3HP unit (0→67 mol%), decreased the X-ray crystallinities (60→7%), indicating the exclusion of 3HP from the crystalline phase of P(3HB). ^{13}C solid-state NMR spin-lattice relaxation time measurements showed that no co-crystallization of 3HP and (*R*)-3HB units occurs in the same crystal lattice in the copolymer [119].

It has also been proved that the increase of the 4HB content (0→49 mol%) decreased the crystallinity of P(3HB-*co*-4HB) copolymer [120, 121]. Only one crystalline form of the P(3HB) lattice was present in the lattice of P(3HB-*co*-4HB) copolymers with ranging compositions of 4HB (0-29 mol%). On the contrary, P(3HB-*co*-4HB) copolymers with ranging compositions of 4HB (78-100 mol%), indicated only the presence of P(4HB) lattice. Additionally, in the case of P(3HB-*co*-4HB), a decrease of the 4HB fraction caused a decrease to the crystal growth rates, implying the exclusion of 4HB units from the crystalline phase of P(3HB) [100, 101].

Recent literature reports on the preparation of single crystals of P(3HB) copolymers with 4HB, (*R*)-3HHx, and 6-hydroxyhexanoate (6HH) units as the second monomer, indicated a lath-shaped morphology of the crystals with a similar electron diffraction pattern to that of P(3HB), and no differences in the *d*-spacing between the P(3HB) and copolymer single crystals, implying the exclusion of the second monomers from the crystal lattice and their existence on the crystal surface [122, 123].

CONCLUSION

PHAs, unlike other biodegradable polymers, attracted the interest in both the scientific community and industry, because of their varied and especially therapeutic applications. Given the shortage and the cost of oil, as well as the harmful effects of petroleum-based plastics, research efforts have focused on the discovery of inexpensive alternative materials with improved properties. As the necessity for: a) cheap carbon sources utilized as raw materials, and b) new microorganisms capable of harnessing these sources through multiple metabolic pathways, has become imperative, research efforts have focused on the development of novel chemical monomeric structures as constituents of PHA polymeric chains. Different metabolic pathways direct the synthetic route of different PHA species. The possibility of producing PHAs consisting of HAs of specific compositions depends highly on the adequate screening of bacteria able to synthesize endogenously precursor substrates from simple and cheap carbon sources such as industrial waste, wastewaters, industrial by-products or agricultural feedstocks. Furthermore, the heterologous expression of PHA synthase structural genes in physiological backgrounds, through anabolic or catabolic pathways, significantly affects the provision of precursor substrates. Moreover, genetic engineering plays an important role in PHA synthase associated site-directed mutagenesis, influencing the diversity of PHA chemical structures. All these synthetic strategies and structural factors described herein contribute in the morphological and chemical diversities of PHAs.

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Publications from the Last 3 Years:

1. Halevas, E., Tsave, O., Yavropoulou, M., Yovos, J. G., Hatzidimitriou, A., Psycharis, V., Salifoglou, A. (2017) *In vitro* structure-specific Zn(II)-induced adipogenesis and structure-function bioreactivity correlations., *J. Inorg. Biochem.*, 177, 228-249.
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5. Halevas, E., Chatzigeorgiou, E., Hadjispyrou, S., Hatzidimitriou, A., Psycharis, V., Salifoglou, A. (2017) pH- and Ligand Structure-Specific Synthesis, Structure-Lattice Dimensionality and Spectroscopic Fingerprint in Novel Binary In(III)-Hydroxycarboxylic Acid Materials., *Polyhedron*, 127, 420-431.
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1. **Patent record available from the European Patent Office (1999)** Composite material based on hexacyanoferrates and polymer, method for making it and use. Publication date: 1999-06-23, Publication number: EP0923412 (A1). Inventors:

Loos-Neskovic; Christiane (Fontenay aux Roses, FR), Vidal-Madjar; Claire (Paris, FR), Dulieu; Jacqueline (Malakoff, FR), Pantazaki; Anastasia (Thessaloniki, GR). Assignee: Commissariat a l'Energie Atomique (Paris, FR) Centre National de la Recherche Scientifique (Paris, FR)

2. **Patent record available from the World Intellectual Property Organization (WIPO)** (Publication date: 1999-01-21, Publication number: WO9902255 (A1). Inventors: Loos-Neskovic; Christiane (Fontenay aux Roses, FR), Vidal-Madjar; Claire (Paris, FR), Dulieu; Jacqueline (Malakoff, FR), Pantazaki; Anastasia (Thessaloniki, GR). Assignee: Commissariat a l'Energie Atomique (Paris, FR) Centre National de la Recherche Scientifique (Paris, FR)
3. **United States Patent No 6,558,552 May 6, 2003.** Composite material based on hexacyanoferrates and polymer, method for making it and use. Inventors: Loos-Neskovic; Christiane (Fontenay aux Roses, FR), Vidal-Madjar; Claire (Paris, FR), Dulieu; Jacqueline (Malakoff, FR), Pantazaki; Anastasia (Thessaloniki, GR). Assignee: Commissariat a l'Energie Atomique (Paris, FR) Centre National de la Recherche Scientifique (Paris, FR).

Publications from the Last 3 Years:

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5. Giannousi K., Menelaou M., Arvanitidis J., Angelakeris M., Pantazaki A.and Dendrinou-Samara C. (2015) Hetero-nanocomposites of magnetic and antifungal nanoparticles as a platform for magnetomechanical stress induction in *Saccharomyces cerevisia*e. *J. Mater. Chem. B,* 3, 5341-5351

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12. Tsitouroudi F., Karatza A., Karoulias S., Pantazaki A., Andriotis E. G. and Choli-Papadopoulou T. (2017) "In cell" biotinylation and immobilization of BMP2 (Bone Morphogenetic Protein 2) on polymeric surfaces. *Biochemical Engineering Journal* 123: 1-12.
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Chapter 5

POLYHYDROXYESTERS AS SCAFFOLDS FOR TISSUE ENGINEERING

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ABSTRACT

Tissue engineering is a field that has gained a lot of advancement since the discovery of biopolymers. Biopolymers are simply polymers that are made-up of polymeric biomolecules. They consist of monomeric units that are covalently bonded to one another in order to form very large structures. Biopolymers have been widely used as biomaterials for the construction of tissue engineering scaffold. Scaffolds have been used for tissue engineering such as bone, cartilage, ligament, skin, vascular tissues, neural tissues and skeletal muscles. Polyhydroxyesters are typical examples of synthetic biopolymers that have been employed for this application. Their exceptional properties, such as: high surface-to-volume ratio, high porosity with very small pore size, biodegradation, and mechanical property have made them gain a lot of attention in this field. Also, they have advantages which are significant for tissue engineering. This chapter focuses on polyhydroxyesters, such as: PLA (Polylactide), PGA (Polyglycolide or poly(glycolic acid)), PCL (Polycaprolactone) and PLGA (Poly(lactide-*co*-glycolide), which have diverse applications in tissue engineering. Details of these polyhydroxyesters and their application in tissue engineering will be discussed in this chapter.

Keywords: biopolymer, scaffold, polyhydroxyesters, tissue engineering

1. INTRODUCTION

Tissue engineering is a field that cuts across other fields in life science. It involves disciplines, such as: engineering, biology and medicine. It basically focuses on the development of biological materials capable of restoring, replacing or enhancing tissues or the entire function of an organ (Langer and Vacanti, 1993). This is accomplished by combining engineering techniques, cells, materials and methods, appropriate biochemical and physiochemical factors that can improve or substitute a biological function. Tissue engineering functions on the principle that cells seeded into three dimensional biocompatible scaffolds are capable of growing into tissues that can function maximally. This is achieved under an environment that permits controlled biomimicry (Shi et al., 2010). In animal tissues, a three dimensional nanofibrous support system, known as extracellular matrix (ECM) surrounds the cell. The support system does not only provide support for the cell, it also directs the behaviour of the cell as well, through cell-ECM interaction (Stevens and George, 2005, Shi et al., 2010). In addition, these scaffolds also function in the storage, release and activation of a broad range of biological factors that assist in cell-cell and cell-soluble factor interactions (Taipale and Keski-Oja, 1997, Shi et al., 2010). For any tissue engineering process to be considered successful, it must develop a scaffold system that closely resembles the ECM in terms of complexity and functionality. Therefore, it is very essential that scaffolds used in tissues engineering be able to completely serve as suitable substitute (Goldberg et al., 2007). Advances in research on biomaterials, stem cells, growth and differentiation factors and an environ-

ment that biomimics, have offered the opportunity to design tissues in the laboratory by combining engineered ECM scaffolds, cells and biomolecules that are active. The great development in this multidisciplinary field has given rise to novel materials for the replacement of tissues (Azimi et al., 2014).

In recent years, biodegradable polymeric materials have been greatly investigated and studied for the design of such scaffolds that can perform excellently, as substitutes. They have gained considerable attention in the field of tissue engineering amongst other biomedical applications. They are used as functional biomaterial in tissue engineering because they are biodegradable, biocompatible and they are able to undergo hydrolysis in the human body (Bikiaris et al., 2007). Thus, they are able to develop a support system that is tissue-specific and complex. Poly(α-ester) is a typical example of this type of polymer. It is a hydrolytically degradable polymer that has a backbone which consist of an aliphatic ester bond. Members of this class of polymer have chemical bonds in their backbone that are hydrolytically liable hence, they can be broken down without secondary influence.

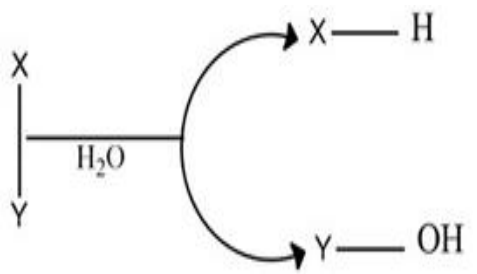

Figure 1. Showing the ester bond as it is cleaved by a molecule of water yielding two products (X-H and X-OH) respectively.

The breakdown of the ester bond contained in PHEs leads to the formation of two species. One product gains a hydrogen atom while the other gains a hydroxyl group. This class of synthetic biodegradable polymers that have been extensively researched it has been discovered that all the commercially available types of PHEs are theoretically degradable. However, due to the fact that the ester bond present in this polymer family are hydrolytically stable, only PHEs with relatively short aliphatic chain length can be employed for use in biomedical applications. Although, these polymers are often slightly hydrophobic, they still undergo bulk erosion as a result of the stability of the ester bond (Ulery et al., 2011, Göpferich, 1997). Biodegradable aliphatic polyhydroxyester such as PLA (Polylactide), PGA (Polyglycolide or poly(glycolic acid)), PCL (Polycaprolactone) and PLGA (Poly(lactide-*co*-glycolide) are some of the biopolymer that have been synthesized and used as scaffolds in tissue engineering. The major feature that makes them function as biomaterial for application in tissue engineering is their relative degradation rates and erosion mechanisms. A number of easy methods have been used in

the synthesis of these three dimensional polymeric scaffold (Kulkarni and Rao, 2013). This chapter focuses on some of these techniques as well as the structure, properties advantages and disadvantages of some of the commonly synthesized various polyhydroxyesters utilized in tissue engineering. Emphasis will also be on the application of these biopolymers in tissue engineering. At the moment, great breakthroughs have occurred and exponential growth in economic activities within the tissue engineering sector are been recorded. For example, the sales of regenerative biomaterials is beyond US$240 million per year (Lysaght et al., 2008).

1.1. Scaffolds and Their Ideal Characteristics

Scaffolds in engineered tissues are materials that are designed to mimic the function of ECM in native tissues of target, at least partially (Chan and Leong, 2008). For over ten years, several scaffolds have been designed for different applications in tissue engineering (De Laporte and Shea, 2007, Frimberger et al., 2006, Harrison et al., 2007, Nakabayashi, 2003, Raghunath et al., 2007). However, for any scaffold to be used in tissue engineering, it is important that the material properties as well as the biological properties must be investigated and examined. Biological testing includes evaluation of the immune and foreign body reactions and the implementation of different cells or growth factors to enhance the properties of the scaffold. Also, the type of application intended for the scaffold must be put into consideration as well as the site of action. For instance, a scaffold intended for the mandible bone must possess strong mechanical properties because it is one of the strongest bones (Earthman et al., 2006). Whereas, the mechanical properties of a scaffold designed for the orbital bone may not really be of great consideration because their mechanical load is not large (Chalasani et al., 2007). Furthermore, in evaluating a scaffold, the exact degradation rate of the material must be studied because natural tissues gradually replaces scaffolds as they degrade. Although, depending on the tissue to be replaced, the degradation rate can be controlled or altered by adjusting its chemical or physical properties. It is important to study the rate of degradation because it affects the generation of by-products of degradation. These by-products have the tendency to disrupt the process of the tissue repair by causing inflammation or preventing the growth of the tissue (Gilbert et al., 2007, Van Amerongen et al., 2006). In addition, depending on the type of application, the surface properties and the ability to design scaffolds into a particular construct is also necessary. In bone tissue engineering, scaffolds used are typically cube or disc-shaped (Hollister et al., 2002). Nerve, vascular and trachea tissue engineering employs tube shaped scaffolds (Widmer et al., 1998) while skin, intestine and liver tissue engineering requires flat matrix shaped scaffolds (Gomes et al., 2001). Scaffolds can also be in form of injectable hydrogels that

can be used to fill defects that have irregular shapes (Thornton et al., 2004). Generally, scaffolds must promote the growth, attachment and migration of cells. Also, they should be able to respond to the changes involved in the growth and development of cells. The porosity of scaffold is of great importance as this helps promote the proliferation and differentiation of cells as well as the exchange of nutrient and metabolites. Furthermore, they must be biocompatible, non-immunogenic, nontoxic and easily manufactured (Patel and Fisher, 2008).

1.2. Types of Scaffold in Tissue Engineering

Materials used in the design and development of scaffolds may be of synthetic or biological origin, it can also be degradable or non-degradable. It largely depends on the application it is intended to be used for (Ramakrishna et al., 2001). They can be classified into different types based on their structural, chemical and biological properties. These properties depends on the composition, structure ad arrangement of the macromolecules that makes up the scaffold. The major materials used as scaffold for biomedical applications include natural polymers, synthetic biodegradable and non-biodegradable polymers (Dhandayuthapani et al., 2011). Hydrogels however, are a type of scaffold that can either be of natural source of synthetic source.

1.2.1. Natural

Natural scaffolds are known to be the first biodegradable and biocompatible materials used clinically as scaffolds (Nair and Laurencin, 2007). This is because of their bioactive properties that makes them have better interactions with the cells. This in turn permits them to enhance the cell's performance in the biological system. Scaffold of natural source (plants or animals) consists of protein or carbohydrates with specific biochemical, mechanical and structural properties (Dhandayuthapani et al., 2011). These scaffolds possess greater advantage because of the multi-functional groups that are present on their surfaces. Hence, they can be tailored for particular applications (Patel and Fisher, 2008). Examples of natural scaffolds that have been used for the repair and construction of damaged tissues are gelatine, collagen (protein) and cellulose, chitin (Ratner et al., 2004, Li et al., 2006c, Patel et al., 2006, Perka et al., 2000, Sumita et al., 2006, Yoo et al., 2005). Porcine collagen is a typical example of commercially available natural scaffold in form of a sheet that is used in tissue engineering in paediatric patients. It was used to seal the anterior wall after a surgery and no immune response was observed (Richards et al., 2005). In another application, hyalomatrix (made up of silicone and hyaluric acid) was successfully used to cover the dermal plane of a patient with burns and a stimulated spontaneous healing was observed. The degradation rate of the matrix was slower

compared to other methods of treatment. This helped in providing sufficient time for the complete healing of the wound (Esposito et al., 2007). Of recent, a scaffold made from human fibrin was used for auricular chondrocyte culture in order to engineer elastic cartilage in a paediatric patient. Studies carried out on the genes showed that the engineered cartilage had the same features with the natural elastic cartilage. Therefore, it can be used as scaffold for ear reconstruction in paediatrics (Richards et al., 2005). Despite the fact that natural scaffolds have been applied in tissue engineering, limitations such as inability to modify their chemical and biological properties for specific applications have caused a shift in focus to synthetic and blended scaffolds (Patel and Fisher, 2008).

1.2.2. Synthetic

Synthetic biomaterials are more favourable for use in tissue engineering, this is because their properties (physical and biological) can be modified. The major biomaterials that belong to this class include: glycolic acid derivatives, lactic acid derivatives, and other polyester derivatives. These biomaterials have been found to be very useful in biomedicine because of these properties (e.g., degradation time, porosity etc.) hence, they are employed for different specific applications (Patel and Fisher, 2008). Copolymerized scaffolds (e.g., L-lactide and ε-caprolactone) of this class of synthetic biomaterials are also employed in biomedicine. In tissue engineering, these synthetic biomaterials are known to facilitate the restoration of functions and structures of tissues that are damaged or diseased. The advantage of this type of scaffold is that their physicochemical and mechanical properties are similar to those of biological tissues (Dhandayuthapani et al., 2011). In addition, they are produced in large, uniform quantities under controlled conditions and they possess longer shelf life compared to the natural scaffolds and they are cheap (Dhandayuthapani et al., 2011, Patel and Fisher, 2008). They possess mechanical and physical properties such as elastic modulus, tensile strength and degradation rate that are predictable and reproducible (Gunatillake et al., 2006).

2. PHEs IN TISSUE ENGINEERING

Amongst all synthetic biomaterials used as scaffold in tissue engineering, PHEs have gained greater attention due to several benefits and advantage associated with their use. They have found diverse application in different areas in tissue engineering. The advantages they have over other biomaterials are attributed to their awesome properties and characteristics which allows wider application in tissue engineering. PLA, PGA, and PLGA copolymers are among some of most commonly used synthetic polymers in tissue engineering (Ma, 2004).

2.1. Structure and Properties

For every biomaterial that is a member of the PHE family, there exist a unique structural formula. The different structural formulas of the four basic members of this family that have been widely exploited in tissue engineering are discussed in this chapter.

2.1.1. PLA

It is made up of monomeric units of lactic acid and it consists of an asymmetrical carbon atom in its structural unit, this gives it the ability to be optically active. PLA possesses chiral molecule hence, it is possible to obtain the isotactic poly(L-lactic acid) (PLLA), poly(D-lactic acid) (PDLA), poly(D,L-lactic acid) (PDLLA) – a racemic mixture of PLLA and PDLA, as well as meso-poly(lactic acid) (Ulery et al., 2011). PLA is one of the few polymers that has stereochemical structure which can easily be altered by polymerizing a controlled mixture its isomers in order to yield an amorphous or semi-crystalline polymers with high molecular weight (Avérous, 2008).

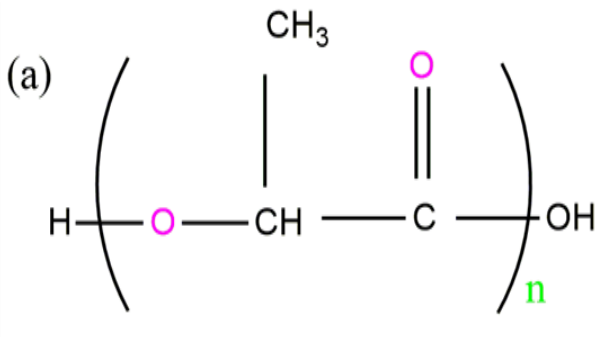

Figure 2. Showing the structure of PLA.

2.1.2. PGA

Poly-g-Glutamic acid (PGA) consists of a homo-polyamide comprizing of D- and L-Glutamic acid monomers. They are linked by an amide linkage formed between the α-amino and the g-carboxyl groups hence, it provides resistance to proteases (Najar and Das, 2015). The polymerization of glutamate through the g-amide linkage makes the polypeptide anomalous. PGA has a chiral centre in each glutamate unit thereby making it optically reactive (Sung et al., 2005, Yoon et al., 2000). Three active stereo chemicals of PGA have been discovered viz; the homopolymer made up of D- Glutamate (D- PGA), the homopolymer of L- Glutamate (L-PGA), and the copolymer composed of D- and L-Glutamate (D-L-PGA) (Sung et al., 2005). The collective name for g-(D)-Poly-Glutamic acid, g-(L)-Poly-Glutamic acid, and g-(D,L)-Poly-Glutamic acid is g-Poly-Glutamic acid (g-PGA) (Ito et al., 1996).

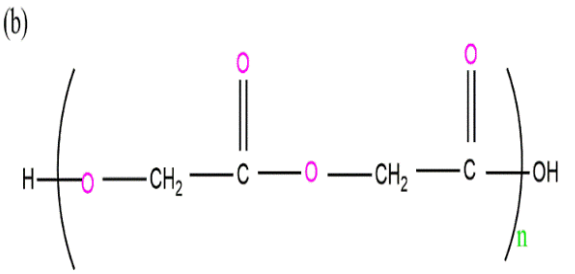

Figure 3. Showing the structure of PGA.

2.1.3. PCL

It is a biodegradable polyester that consists of monomers of ε-caprolactone or simply caprolactone. Caprolactone is a lactone (cyclic ester) that has a seven membered ring and its name is gotten from caproic acid. PCL consists of hexanoate repeat units, five methylenes [$(CH_2)_5$] and an ester functional group [CO_2] as the repeating unit (Hernández et al., 2013). The presence of the CH_2 moities in its repeating units affects its degradation rate, causing it degrade slowly (Bhattarai et al., 2006, Hao et al., 2002). It exists as a semicrystalline form because the structure is regular. The functional groups attached to PCL also makes it more hydrophilic, adhesive, or biocompatible thus favours cell responses (Woodruff and Hutmacher, 2010).

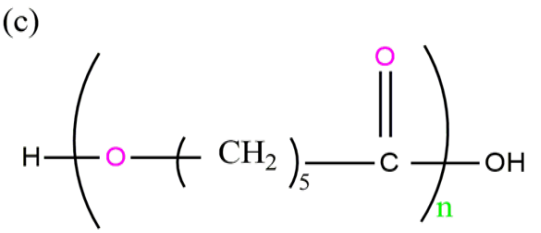

Figure 4. Showing the structure of PCL.

2.1.4. PLGA

PLGA however, is a copolymer of two members of the PHE family viz; polylactic acid (both L- and D- lactide forms) and polyglycolic acid (PGA). The copolymerization of lactic acid and glycolic acid at different ratios gives rise to different forms of poly(lactide-*co*-glycolide) PLGA (Nimesh, 2013). PLGA exists as d, l, and dl isomers because of the presence of a pendant methyl group on the alpha carbon of PLA (PLA becomes chiral molecule). Although, most studies do not provide the details of the exact stereochemistry of PLGA and this plays a very important role in the properties of the copolymer. The presence of the free carboxyl end-group in PLGA allows for the chemical modification of the degradation rate or other properties. An example of such

modification is the covalent bonding of the free carboxyl end group present in PLGA with amine groups. Thus, forming a covalent amide bond for a more enhanced application of PLGA (Lanao et al., 2013).

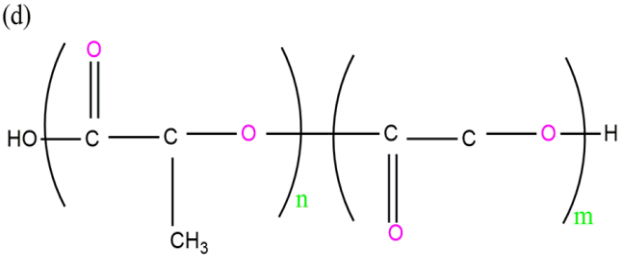

Figure 5. Showing the structure of PLGA (The suffixes n and m represent the number of lactic and glycolic acid respectively).

2.1.5. Physical, Chemical and Mechanical Properties

The properties that are more intensively studied include: density (p, in g/cm3), tensile strength (σ, in MPa), tensile modulus (E, in GPa), ultimate strain (in %). By dividing the original property by the polymer density, specific tensile properties are also obtained thus, specific tensile strength (s*, in Nm/g) and specific tensile modulus (E*, in kNm/g) are also studied. In addition, the characteristic temperatures such as glass transition temperature (T_g, in °C) and melt point (T_m, in °C) are also investigated (properties). The table below shows the summary of different properties of some common PHEs and their limits (upper and lower) (Brandrup and Immergut, 1989, Van de Velde and Kiekens, 2002, Jacobsen and Fritz, 1999, Middleton and Tipton, 1998, Zein et al., 1999).

Table 1. Properties of some common PHEs

Type of biopolymer	**Properties and their limits**							
	ρ (g/cm^3)	σ (MPa)	*E*(GPa)	ε (%)	σ* (Nm/g)	*E** (kNm/g)	T_g (°C)	T_m (°C)
PCL	1.11** 1.15*	20.7** 42.0*	0.21** 0.44*	300** 1000*	18.6** 36.7*	0.19** 0.38*	-60** -65*	58** 65*
PGA	1.50** 1.70*	60.0** 99.7*	6.00** 7.00*	1.5** 20.0*	40.0** >45.1*	4.00** 4.51*	35** 45*	220** 233*
PLA	1.21** 1.25*	21.0** 60.0*	0.35** 0.30*	2.5** 6.0*	16.8** 48.0*	0.28** 2.80*	45** 60*	150** 162*
PLGA	1.30** 1.40*	41.4** 55.2*	1.00** 4.34*	2.0** 10.0*	30.9** 41.2*	0.77** 2.14*	40** 50*	am

NB: am (amorphous hence no melting point)

** (upper limit)

* (lower limit).

2.2. Synthesis of Biodegradable PHEs in Tissue Engineering

Polyhydroxyesters are degradable biomaterials that have been heavily researched. This is because they are relatively easy to synthesize. The major methods of synthesizing polymers that belong to this family are condensation polymerization (direct or azeotropic dehydration) and ring-opening polymerization (Ulery et al., 2011, Coulembier et al., 2006).

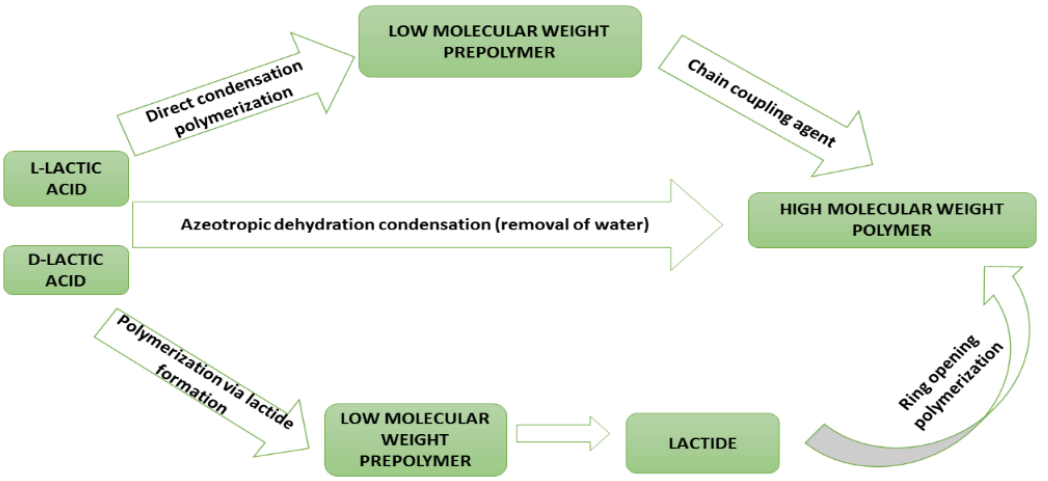

Figure 6. Showing the methods of synthesizing high molecular weight PHEs, (using the synthesis of PLA as a case study).

The synthesis of PLA involves several steps that begins from lactic acid production, formation of a transitional product (lactide) and eventually ends with the polymerization of the produced lactic acid (Garlotta, 2001, Hartmann, 1998, Auras et al., 2004, Mehta et al., 2005, Södergård and Stolt, 2002). However, the entire process of synthesizing PLA can follow three major routes (as seen in Figure 6 above). The first route is the direct condensation of lactic acid to produce a brittle polymer with low molecular weight. This product is often unusable but by coupling external agents, the chain length can be increases hence, it becomes useful. The second route involves the azeotropic dehydrative condensation of lactic acid to yield PLA with high molecular weight that does not require external agents to extend its chain length (Hartmann, 1998). The last and major route is the ring opening polymerization of lactide (cyclic dimmer of lactic acid) in the presence of a catalyst. This results in PLA with very high molecular weight (Doi and Steinbüchel, 2002, Kalia and Avérous, 2011, Lim et al., 2010). This method consists of three steps namely; polycondensation of lactic acid monomers to low-molecular weight PLA, depolymerization of the PLA into the lactide, and catalytic ring-opening polymerization of the lactide intermediate resulting in PLA with a controlled molecular weight (Kim et al., 2009). Although, lactic acid can serve as copolymer to obtain a more

complex macromolecule. Of all the range of polymerization processes used for the synthesis of PLA, polycondensation (solution and melt polycondensation) are the least expensive routes but these routes do not produce solvent-free high molecular weight PLA easily (Hamad et al., 2015). While ring opening polymerization on the other hand is expensive due to the additional complicated purification steps needed. The advantage of ring opening polymerization process is the ability to control the molecular weight of the fabricated PLA via temperature, type of catalyst etc. Also, it is possible to control the ratio and the sequence of D- and L-lactic acid units in the final polymer (Gupta et al., 2007). Similar to PLA, PLGA is synthesized via ring-opening polymerization of lactide and glycolide. This is achieved in the presence of a catalyst (metal. PLGA of a wide range of molecular weight can be synthesized by this route) (Nimesh, 2013). This copolymer can be obtained by using different ratios of its constituent monomers lactic and glycolic acid. This method allows for alteration of the properties (e.g., physical and biodegradation rate) of PLGA by changing the amount of the monomeric unit present in the final copolymer (Fonseca et al., 2015). Both low and high molecular weight PLGA can be synthesized by using this method, using catalysts such as tin (II) alkoxides, or aluminum isopropoxide or using cyclic dimers as a starting material (Lü et al., 2009). The process involves the linkage of monomeric units of glycolic or lactic acid successively by ester linkages. This results to the formation of a linear, amorphous aliphatic random polyester PLGA (Lanao et al., 2013). However, this method of PLGA synthesis limits the number and types of sequences obtainable hence, randomly distributed atactic or syndiotactic PLGAs are obtained (Dechy-Cabaret et al., 2004). PCL was investigated as early as 1930s and in the same manner like other polyhydroxylesters discussed above, it is synthesized via ring opening polymerization of ε-caprolactone (cyclic monomer) (Woodruff and Hutmacher, 2010). Of recent, various types of catalyst required for the synthesis of PCL is studied and reviewed (Labet and Thielemans, 2009). It has been discovered that catalysts such as stannous octoate used in the polymerization of alcohols with low molecular weight can be used to control the molecular weight of PCL as well (Storey and Taylor, 1996). Various mechanisms such as anionic, cationic, co-ordination and radical affects the polymerization of PCL. This is seen in the molecular weight, molecular weight distribution and end group composition of the resultant PCL (16). Although, PCL samples have an average molecular weight of between 3,000 to 80,000 and they are graded based on their molecular weight (Hayashi, 1994).

2.3. Fabrication of PHE Scaffolds in Tissue Engineering

In order to engineer functional tissues and organs scaffolds, it is important to understand the fabrication techniques that is suitable to produce the desired feature for the specific application. Different methodologies are employed for the fabrication of

scaffolds that will facilitate the distribution of cells and guide their growth into three-dimensional space. The main techniques used for the fabrication of scaffolds fall under two categories which are non-designed and designed manufacturing techniques (Subia et al., 2010). Non-designed manufacturing techniques includes: freeze drying or emulsion freezing (Whang et al., 1995), solvent casting or particulate leaching, phase separation (Zhao et al., 2002, Liu and Ma, 2004, Ma, 2004), gas foaming or high pressure processing, melt moulding, electrospinning and combination of these techniques. Designed manufacturing technique includes rapid prototyping of solid free-form technologies (Sultana et al., 2015). A few of the commonly applied techniques for the fabrication of PHEs of interest in this chapter are explained below.

2.3.1. Electrospining

It is a very versatile technique that is not invasive and does not need the application of coagulation chemistry or high temperature for fibre generation (Subia et al., 2010). It is a technique that is used to manufacture scaffold with diameters in the nanoscale of less than 1000 nm or microscale of greater than 1 μm range (Reneker and Chun, 1996). Fibres within this range are formed by regulating some parameters such as polymer solution properties (e.g., viscosity and conductivity) and operational conditions (e.g., strength of the electrical field applied and hydrostatic pressure in the capillary tube (Lu et al., 2013). Poly(lactic acid) [PLLA], poly(glycolic acid), poly(lactic-co-glycolic acid) [PLGA] and polycaprolactone are some examples of polymers used in tissue engineering that have been fabricated by using electrospinning method (Li et al., 2012, Park et al., 2012, Chakrapani et al., 2012). This technique is quick and simple for the manufacture of different types of nanofibrous scaffold but it is still challenging to fabricate scaffolds with complex structures for some tissue engineering application (Lu et al., 2013). A major advantage of this technique is the ability to fabricate scaffold that have structural features that is compatible for cell growth and tissue formation (Li and Tuan, 2009, Liang et al., 2007, Leong et al., 2009). This enables supports cell growth *in-vio* and *in vitro* because they influence the adhesion, expression of cells and also the transport of oxygen and nutrients to the cells (Subia et al., 2010). Although, cell seeding remains the problem associated with this technique, however, this is overcome by cryo-spinning, thereby allowing the formation of hole of desired size in the electron spun matrices (Baker et al., 2008, Leong et al., 2009).

2.3.2. Phase Separation

This is a process that is induced either thermally or by non-solvents and it is used to fabricate porous membranes for the purpose of separation or filtration (Nolsøe et al., 2007). Usually, a heterogeneous pore structure is the product obtained from non-solvent induced phase separation process and such scaffold is not suitable for tissue engineering application that requires a structure with uniform pore (Guillen et al., 2011). Thermally

induced phase separation however, results to the formation of a polymer rich domain and a polymer-lean domain which solidifies to form a matrix and pore (due to solvent removal) respectively. Phase separation induced thermally can be further divided into solid-liquid and liquid-liquid phase separation. The former is used for inducing solvent crystallization from a polymer solution which leads to the formation of a structure with pore while the latter forms a structure with a polymer-rich and a polymer-lean phase (Ma, 2004, Ma and Zhang, 1999). Examples of a polymer used in tissue engineering that has been fabricated with this method are PCL, PLGA and PLLA (a form of PLA) scaffold. This was achieved by using liquid-liquid phase separation (Ma et al., 2001). Different scaffolds with varying morphologies can be fabricated by adjusting parameter such as polymer concentration and cooling rate during the process of fabrication (Nam and Park, 1999, Schugens et al., 1996a, Schugens et al., 1996b). The advantage of this technique is the ability to combine easily with other techniques employed for fabrication of scaffolds especially particulate leaching and rapid prototyping. This combination is capable of fabricating fibrous scaffolds that can be employed in tissue engineering (Smith et al., 2007).

2.3.3. Solvent Casting/Particulate Leaching

This is one of the common and easy technique, done by casting a polymer solution into a salt (porogen)-filled mold. It is a technique that has been broadly employed in the fabrication of scaffolds such as PCL, PLGA, PLA and PGA in tissue engineering application (Ma and Langer, 1999, Lu et al., 2000). The size of the pore formed can be controlled by adjusting the size of the salt crystal used as well as the ratio of the polymer/salt. However, variables such as the shape and the inter-pore openings are uncontrollable but with the development of new technologies, this limitation is overcome (Ma, 2004). The advantage of this method is that a less quantity of the polymer is required to fabricate its scaffold (Subia et al., 2010). Also this technique has helped to overcome challenges associated with the fibre bonding technique (Sultana et al., 2015).

2.3.4. Fibre Bonding

This technique was designed by Mikos and his co-workers (Mikos et al., 1993). In their experiment, PLLA was dissolved in chloroform, followed by the addition of a mesh of PGA (non-woven mesh). The evaporation of the solvent resulted in the formation of a composite material consisting of a non-bonded PGA fibre in a PLLA matrix (Chen et al., 2001). In the course of the post treatment, the fibre bonding occurred when the temperature was above the melting temperature of PGA. This process yielded a PGA scaffold bonded by heat treatment (Subia et al., 2010). The resultant product (scaffold) helps in providing mechanical support and stability to tissues as well as permitting their growth. A major advantage associated with the use of this technique is the provision of large surface area suitable for scaffold application. Thus, a larger surface area for cell

attachment, sufficient space for regeneration of ECM is made available (Moroni et al., 2008).

2.3.5. Freeze Drying

This is another fabrication technique based on the principle of sublimation, and it leads to the formation of porous scaffolds that are usable in tissue engineering applications (Schoof et al., 2001, Whang et al., 1995). A porous scaffold with high porosity and inter connectivity is obtained by dissolution of polymer in a solvent, followed by freezing the solution and subsequently removing the solvent by lyophilization under high vacuum (Mandal and Kundu, 2009a, Mandal and Kundu, 2009b). Polymers such as PGA, PLLA, PLGA, PLGA/PPF blends are fabricated via this technique (Altman et al., 2003, Vepari and Kaplan, 2007). Control of the pore size of the scaffold is possible by adjusting the freezing rate and pH, the faster the freezing rate, the smaller the pore size. Also, the control of the solidification in one direction has led to the production of a homogenous 3D-pore structure (Schoof et al., 2001). The advantage of this method is that it does not require high temperature neither does it require a separate leaching process. However, the limitation is the small pore size and long fabrication time (Boland et al., 2004).

2.4. Functions and Biodegradation

2.4.1. Comparison between the Functions of Extracellular Matrix (ECM) and Scaffolds

In view of the fact that the main aim of developing scaffolds is to adequately mimic the function of ECM as earlier stated, all scaffolds employed in tissue engineering must be very similar in structure and function to those of the targeted native tissue. The table below (Table 2) therefore shows the similarities in the function of scaffolds in engineered tissues and that of ECM in native tissues (Chan and Leong, 2008).

2.4.2. Biodegradation

Biomaterials that are degradable have changed the application of materials such as PHEs for tissue engineering application. Generally the degradation of polymeric materials involves the cleavage of bonds present in the polymer that are either hydrolytically or enzymatically sensitive. The degradation of scaffolds can take place via physical or chemical or biological mechanisms (Dhandayuthapani et al., 2011). In the biological process, biological agents such as enzymes are involved (Vacanti and Langer, 1999). However, the rate of the polymer's biodegradation depends on factors such as properties of the polymer, chemical structure, degree of hydrophobicity or hydrophilicity, glass transition temperature etc. (Ye et al., 1997). Biodegradable scaffolds degrades

gradually until they are replaced by the newly grown tissues from the attached cells (Vacanti and Langer, 1999). During degradation, the scaffolds is undone (dismantled) and material dissolution/resorption takes place (Middleton and Tipton, 2000).

Table 2. Showing the functions of ECM and scaffolds in natural and engineered tissues respectively

Analogous function of scaffolds in engineered tissues	**Function of ECM in natural tissues**
They serve as delivery devices and reservoir for growth-stimulating factors that are exogenously applied	ECM serves as reservoirs of growth factors and also potentiates the actions of growth factors
They provide structural support for growth, attachment, migration and differentiation of exogenously applied cells *in vitro* and *in vivo*	ECM provides structural support for cells to live
They provide the mechanical stability and shape to the defected tissue	ECM enhances the mechanical properties of tissues
During remodelling of tissues, scaffolds provides a void volume for vascularization and new tissue formation	ECM provides a flexible physical environment that permits remodelling in response to tissue dynamic processes
Scaffolds interacts with cells actively to promote activities such as cell proliferation and differentiation	ECM provides bioactive cues for adequate response by cells to their microenvironment

It is important that biodegradable materials degrades at the rate that matches the growth of tissues both *in vivo* and *in vitro*. The degradation of polymeric scaffolds results into a breakdown of the internal structure of the scaffold leading to a reduced molecular mass (Woodruff and Hutmacher, 2010). This can be likened to the dissolution of soap, whereby the rate of surface degradation is constant hence, the bulk structure of the scaffold is maintained even though the size is smaller (Dhandayuthapani et al., 2011). This type of degradation gives room for longer mechanical stability for tissue regeneration.

The breakdown of PLGA is by the hydrolysis of ester bonds yields metabolites (its monomeric units) that already exist in the body, that is lactic and glycolic acid which are removed by Kreb cycle (Nimesh, 2013, Mundargi et al., 2008, Ratner et al., 2004). Due to the fact that these metabolites normally take part in some physiological and biochemical pathways, there is very little toxicity associated with the use of PLGA in tissue engineering. For example lactic acid is converted into glucose via Cori cycle and the glucose is in turn used as a source of energy by the body (Mahapatro and Singh, 2011). Factors such as molecular weight, co-polymer composition, stereochemistry, end-group functionalization etc. can affect the degradation of PLGA (Yoshioka et al., 2008).

Although, the degradation of PLGA is not limited to the surface of the device and four consecutive steps are observed during the degradation of this biomaterial (Wu and Wang, 2001). These are:

1) Hydration: it involves the penetration of water into the amorphous part of the polymer, causing a disruption of the van der Waal force and hydrogen bonds, then subsequently a decrease in the glass transition temperature.
2) Initial degradation: it involves the cleavage of covalent bonds, this leads to a decrease in the molecular weight of PLGA.
3) Progressive degration: This involves autocatalysis of the degradation process by the carboxylic end groups as a result of the hydrolytic reaction. A mass loss occurs due to the massive cleavage of the covalent bonds that holds the backbone. This causes a loss of integrity of the biomaterial.
4) Solubilization: This involves further cleavage of the PLGA fragments to molecules that are soluble in the aqueous environment.

In the same manner, PGA and PLA degrades by the hydrolysis of the ester bonds. However, the additional methyl group present in the polymer makes it more hydrophobic hence, it is more stable and resist hydrolysis more than PGA. In addition, PLLA (which is a form of PLA) is known to have slow degradation time and from an *in vivo* research, PLLA of high molecular weight takes more than five years to be completely resorb (Suuronen et al., 1998). Currently, for the degradation time to be reduced, scientists have developed modification techniques that allows for faster degradation of PLLA or copolymerize PLLA with other polymers that are degradable. A type of this modification technique is the use of radiation, this causes the creation of free radicals in the ester alpha carbon, thereby reducing the polymer backbone by removing the ester bond and subsequently, releasing carbon dioxide. (Loo et al., 2005, Tan et al., 2009, Ulery et al., 2011). Generally, the recombination of free radicals causes branching and crosslinking which eventually leads to a decrease in the crystallinity of the polymer. The shortening and decrease in the crystallinity facilitates the rapid degration of PLLA (Ulery et al., 2011).

PCL degrades slowly under normal physiological conditions by the hydrolysis of the ester linkages and releases products that are less acidic compared to PLA or PLGA (Saltzman, 2001). The product of its degradation has minimal or no toxicity hence, its wide application in tissue engineering as long term implantable devices (Mahapatro and Singh, 2011). However, the copolymerization of ε-CL with LA or GA leads to a faster rate of degradation (Fonseca et al., 2015). PCL degradation involves a first step non-enzymatic hydrolytic cleavage of the ester group and a second step intracellular degradation process. In an experiment, it was observed that intracellular degradation occurs to polymers with very high crystallinity and low molecular weight (less than

3000) (Woodward et al., 1985). This agrees with the theory of complete resorption and degradation of PCL with molecular weight of 3000 or less. The mechanism of PCL degradation can be attributed to a random hydrolytic chain scissors of the ester linkages which leads to reduced molecular weight. The average degradation time of PCL is between two to four years depending on the molecular weight of the starting material (Gunatillake and Adhikari, 2003, Middleton and Tipton, 2000).

2.5. Application of PHEs in Tissue Engineering

In order for PHEs to be used to meet the diverse needs in tissue engineering, it is desired that that they possess the following characteristics: (i) structures must be three dimensional and very porous with a pore network that is interconnected to allow for the flow and transport of nutrients and metabolic waste as well as cell growth; (ii) surface must be suitable for the chemistry of call attachment, proliferation and differentiation; (iii) they must be biocompatible and bio-resorbable; (iv) their rate of degradation and resorption must be controllable to match the growth of cells/tissue either *in vitro or in vivo*; and (v) they must possess mechanical properties similar to that of the specific site of application (Azimi et al., 2014, Hutmacher, 2000). The basic steps involved in tissue engineering that employs the use of scaffolds is shown in (Figure 7) below.

PHEs have been used for different applications in tissue engineering. Some of such applications include: cartilage, bone, breast, nerve and skin, blood vessel, cardiovascular, tendon and ligament tissue engineering. A few of these applications are discussed below.

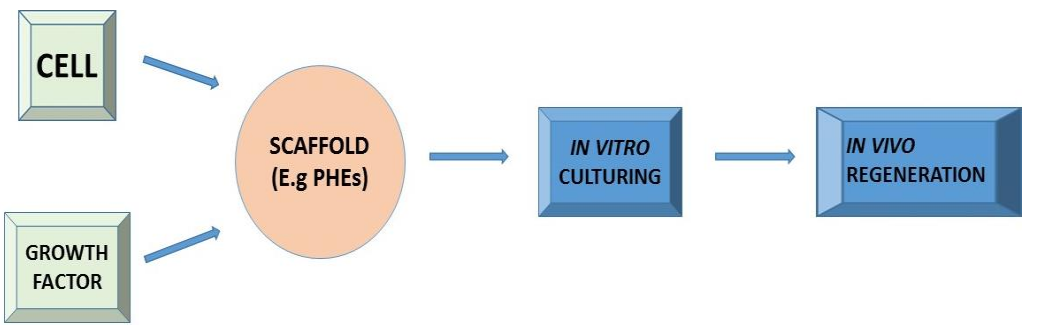

Figure 7. Showing the simple steps in tissue engineering.

2.5.1. Cardiovascular Tissue Engineering

Tissue engineering has been employed to fix the major failures (poor durability and thrombogenicity) of currently available valve (mechanical or biological) prostheses. This technology has been able to grow, repair and remodel cardiovascular tissue at the same time eliminating the disadvantages associated with the use of conventional valve prostheses. The valves in the body that are often replaced are mitral and aortic. Therefore,

to design an aortic valve, a strong scaffold is a required prerequisite because the scaffold on which cells are seeded gives the particular valvular shape to the tissue that is being grown. The scaffold also guides the development and provide support against mechanical forces to the growing tissue (Woodruff and Hutmacher, 2010). In an investigation carried out by Van Lieshout and co, two different scaffold for tissue engineering of the aortic valve were developed. The first was an electrospun valvular scaffold while the other was a knitted valvular scaffold. They reported that the best scaffold for cardiovascular application is that which combined the strength of the knitted structure and the cell-filtering ability of the spun structure (Lieshout et al., 2006, Van Lieshout et al., 2006, van Lieshout, 2005). In addition to the application of PHE scaffold in cardiovascular tissue engineering, PCL scaffold was used as a medium for the cell delivery of human venous myofibroblastas cell. An optimal cell delivery was observed (Balguid et al., 2008). Furthermore, a number of scaffolds have been used to reconstruct valves and vessels to replace the damaged vessels and heart valves. In recent studies, focus has been on applying cell-scaffold matrix for cardiovascular tissue engineering. An example is the culturing of bone marrow-derived mesenchymal cells on a polyglycolic acid and poly-4-hydroxybutyrate scaffold for the development of a tissue-engineered trileaflet heart valve. Results from the histology showed that there was increased confluency of the cells on the scaffold and the biomechanical properties were like that of the native heart valve leaflets (Perry et al., 2003). Another research which involved the seeding of umbilical cord blood derived endothelial progenitor cells on PGA vascular scaffold was done. Analysis carried out revealed that the cell-polymer attachment increased likewise proliferation. Immunohistochemistry result showed the expression of endothelial phenotype (Schmidt et al., 2004). It was concluded that cell-scaffold technique may possess a potential treatment alternative for congenital defects repairs.

2.5.2. Skin Tissue Engineering

Most research on skin tissue engineering focuses on the treatment of second degree burns as they constitute a large number of deaths related to skin injury (Patel and Fisher, 2008). Fabricated scaffolds such as collagen used in engineered human skin are observed to possess poor mechanical properties. Likewise, autographs applied for the treatment of skin burns do not lead to a complete or perfect repair. More studies are therefore undertaken in order to overcome these challenges encountered. One of such studies that have proven to be successful is that carried out by Powell and Boyce (Powell and Boyce, 2009). In their experiment, PCL was blended with collagen and electrospun and a submicron fibre was formed. Test carried out on the scaffold showed that there was improved mechanical strength and stiffness of a cellular construct. Dai and his fellow researcher also developed a composite tissue engineered skin substitute that consisted of

PHE. From the result of their *in vitro* investigation, it was discovered that the scaffold had good cell attachment and proliferation of fibroblasts and keratinocytes. *In vivo* studies was further carried out, they observed that the wound closure rate was faster and a good epidermal differentiation was noticed as well (Dai et al., 2009). Scientist like Reed and his colleagues have also investigated the possibility of applying PHE scaffold for skin tissue engineering. He used different types of cell such as human foreskin fibroblasts, murine keratinocytes and periosteal cells on PHE nanofiber with the aim of designing a trilaminar construct, resembling a compound tissue (Reed et al., 2009). Other research groups such as Chen (Chen et al., 2009), Ananta and her co-workers (Ananta et al., 2008) have investigated the applicability of various types of PHE in skin tissue engineering. Thus far, it appears that PHE scaffold are promizing biomaterials as scaffold for the treatment and management of skin issues in tissue engineering.

2.5.3. Bone Tissue Engineering

Scaffolds with potential ability for bone repair are have been researched and currently, they are still being investigated by different research group (Gough et al., 2004). In a recent study, a scaffold construct made of PLA plates was tested for the repair of skull defects in thirty-four children. Results after implantation showed that there was no dislodgement, infection, or formation of local hematomas or seromas neither was there complications or failures after surgery in any patient (Cohen et al., 2004). In another study, complex tissues such as spongy bone was produced by composite scaffold with a well-organized structure. This was achieved by embedding PLLA fibre in a porous matrix of PCL by using phase inversion/particulate leaching technique and filament winding technology, a porosity of about 80% was obtained with pore size of diameter ranging from 10 and 200μm. No degradation was observed *in vitro* after 35 days in PBS (phosphate buffer solution) however, in NaoH, a significant degradation characterized by loss in weight was observed. When cells (marrow stromal cells and human osteoblasts) were seeded and had reached its plateau at three weeks, a rapid proliferation on the scaffold was observed. This promoted the oriented migration of the bones cells around the scaffold fibre. Thus, the conclusion that the PCL/PLLA scaffold is promizing for bone tissue engineering application. However, it is key that certain parameters such as degradability and cell-material interaction are simultaneously controlled and guided respectively (Guarino et al., 2008). Furthermore, bone research at National University of Singapore gained clinical approval (*in vivo* and *in vitro*) in 2008 for commercialization under the trade name Osteopore™. Another product that has been patented and used in a number of orthopedic applications is the Artelon® specifically Artelon® CMCSpacer Arthro. It comprises of resorbable PCL, it is a T-shaped device used to separates the trapezial and the metacarpal bone of the carpometacarpal joint. (Woodruff and Hutmacher, 2010).

2.5.4. Cartilage Tissue Engineering

The degeneration of the cartilage as a result of congenital abnormalities and trauma has posed a clinical challenge because of the limitation of the basic healing potential of cartilage tissue (Woodruff and Hutmacher, 2010). The local chondrocyte is unable to completely repair the broken cartilage tissue because of the lack of blood supply and subsequent wound healing response, damage to cartilage alone, or chondral lesions. In most cases such as osteoarthritis, in order to completely prevent continuous degeneration of the joint, surgery is the only alternative. Tissue engineering therefore aims to provide satisfactory treatment for such challenges through the design of scaffolds that are biocompatible, structurally and mechanically stable. Also, these scaffolds must have suitable cell source that can be loaded with biomolecules which will facilitate the growth and differentiation of the cell (Tuli et al., 2003). After a study by Huang and his co-workers using a scaffold from the PHE family, it was concluded that the scaffold could successfully recruit mesenchymal cells. It was further observed that chondrogenesis took place when the scaffold was implanted subperiosteally (Huang et al., 2002). Generally, due to the similarity in the morphology of scaffolds to natural extracellular matrix, they have been considered as good candidate for delivery cells in tissue engineering applications. A number of studies have employed nanofibrous PHE scaffold for cartilage tissue engineering (Li et al., 2006b, Li et al., 2005). Additionally, Li and his colleagues assessed a three-dimensional nanofibrous PHE to observe its ability to preserve chondrocytes in a mature functional state. Reports showed that the scaffold facilitated cell proliferation and maintained the phenotype of the chondrocyte. Hence, the scaffold was considered suitable for cartilage tissue engineering (Li et al., 2003). This group of scientist also investigated the potency of nanofibrous PCL scaffold for the repair of cartilage in swine model. At the end of their experiment, no immune reaction was evident and a xenogeneically derived regenerated cartilage was observed (Li et al., 2009).

2.5.5. Tendon and Ligament Tissue Engineering

A successful attempt has been made for the reconstruction of tendon by using PHE scaffold. A good recovery of the functionality of the tendon was reported when PCL scaffold was used to repair gaps in Achilles tendons in a rat model. All the groups that were subjected to the scaffold that was employed for treatment showed a significant improvement at day 28 compared to the untreated rat group (Kazimoğlu et al., 2003). In another experiment, a commercially available PHE scaffold was sutured over ruptured tissue to serve as reinforcement during torn tendon repair. It was observed that the implant (scaffold) maintained its strength as well as elasticity for many years thus, it provided a long term support for the soft tissue. Also, it permitted the tissue's in-growth and remodelling (Woodruff and Hutmacher, 2010). Mesofol® is yet another commercial product that have been applied in tendon tissue engineering. It is often inserted in between muscles or muscles-tendons/nerves to avoid adhesion after operation. It is a

medical device that consists of polylactide and polycaprolactone. It can also be used to fascia's sliding function, by improving the physiologic free gliding thereby, causing a reduction in pains after operation (Klopp et al., 2008).

2.6. Specific PHEs and Their Application in Tissue Engineering

2.6.1. Polylactide (PLA)

In respect to use in the field of biomedicine, only PLLA and PDLA have been studied in-depth and shown to be most promizing (Middleton and Tipton, 2000). PLLA has been widely used in tissue engineering as scaffolds for bone, (Chang et al., 2007, Schofer et al., 2008, Shim et al., 2010, Cai et al., 2010) cartilage, (Ju et al., 2008, Tanaka et al., 2010) tendon, (Tanaka et al., 2010) neural, (Hu et al., 2010, Wang et al., 2010a) and vascular (François et al., 2009) regeneration. A group of scientist have successfully designed PLLA-based patient specific scaffolds (Smith et al., 2009). They were able to convert a CT image of a digit via printing layer-by- layer to a three dimensional PLLA structured wax mold scaffold. They achieved this using a solvent extraction process and paraffin sphere. They were also able to control the nano, micro and macro structure of the scaffold as desired for a wide range of applications. PDLLA is also another polymer obtained by copolymerizing isomers of PLA and it has found application in tissue engineering scaffolds (Hasegawa et al., 2007, Carletti et al., 2011, Yu et al., 2010). Similar to PLLA, it can be combined with polymers such as poly(lactide-*co*glycolide), (Leung et al., 2008) poly(ethylene glycol), (Tsallas et al., 2011, Seck et al., 2010) and chitosan (Cai et al., 2007) to produce composites properties that are specific and desired for a particular application in tissue engineering.

2.6.2. Polyglycolide or Poly(Glycolic Acid (PGA)

PGA is one of the degradable polymers that was first studied for biomedical use (Maurus and Kaeding, 2004). Between the years 1984 to 1996, PGA under the trade name Biofix® was marketed and used as a bone pin for internal application. However from 1996, because poly(L-lactide) is more stable, Biofix converted to poly(L-lactide) base scaffold for internal bone pin (Katz and Turner, 1970, Burns, 1995, Reed, 1999). Recently, focus is on the short term use of PGA in tissue engineering. This is as a result of its rapid degradation and insolubility in several common solvents. PGA is often designed as a mesh network that can be used as a scaffold for bone (Endres et al., 2007, Wang et al., 2010b, Dunne et al., 2010, Pihlajamäki et al., 2010), cartilage (Erggelet et al., 2009, Frisbie et al., 2009, Mahmoudifar and Doran, 2010), tendon (Pihlajamäki et al., 2007, Xu et al., 2010), tooth (Ohara et al., 2010), vaginal (Sayasneh and Johnson, 2010), intestinal (Aysan et al., 2010), lymphatic (Ting Dai et al., 2010), and spinal regeneration (Abbushi et al., 2008).

2.6.3. Polycaprolactone (PCL)

Although, PCL's application in biomedicine is generally limited due to very slow rate of degradation *in vivo*. However, it has found significant application in tissue engineering in numerous ways. The low tensile strength but high elongation at breakage of PCL have made them excellent elastic biomaterial for use in tissue engineering (Gunatillake et al., 2006). The method of synthesizing PCL gives room for the design of scaffolds that consists of adhered microspheres (Garkhal et al., 2007, Danhier et al., 2009) electrospun fibers (Li et al., 2006b, Luciani et al., 2008, Chung et al., 2010). PCL and its various composites have been employed in tissue engineering scaffolds for bone, (Pankajakshan et al., 2008, Plikk et al., 2009, Zuo et al., 2010) ligament (Mountziaris et al., 2010, Surrao et al., 2010), cartilage (Vaquette et al., 2010, Li et al., 2006a), skin (Chung et al., 2010, Garkhal et al., 2007, Li et al., 2009), nerve (Guarino and Ambrosio, 2008, Jeong et al., 2008, Nisbet et al., 2009) and vascular tissues (Liu et al., 2011, Heydarkhan-Hagvall et al., 2008) regeneration. In recent years, advancement in the use of PCL in tissue engineering has been reported. Lee and his colleagues used PCL hybrid in interfacial tissue engineering. They proved that complex tissue interfaces such as bone-ligament interface can be regenerated by seeding distinct scaffold regions with appropriate cells that were harvested from the cartilage or ligament source (Lee et al., 2011).

2.6.4. Poly(Lactide-Co-Glycolide (PLGA)

PLGA is a common example of a degradable copolymer of PHEs that have been broadly studied for its biomedical application. It is currently used as scaffold in tissue engineering for diverse purposes because of its excellent cell adhesion and cell proliferation properties. PLGA has been formed into scaffolds via several techniques which include gas foaming (Chung et al., 2008, Zhu et al., 2008), microsphere sintering (Simpson et al., 2008, Jabbarzadeh et al., 2008, Spalazzi et al., 2008), porogen leaching (Arnold et al., 2007, Narayan and Venkatraman, 2008, Ren et al., 2007), electrospinning (Bashur et al., 2006, Kumbar et al., 2008, Moffat et al., 2008, Aviss et al., 2010), and a novel polymer printing (Ge et al., 2009, Lee et al., 2008) or a combination of these techniques (Leung et al., 2008, Lee et al., 2008, Yoon et al., 2006, Perron et al., 2009) for the creation of a unique nano- and micro-structured materials capable of facilitating the development of tissues. A major advantage of PLGA is that it is comprised of the different properties of PGA and PLA hence, it can be optimized for the desired intended application. The polymer printing technique seems to be promizing in the design of scaffolds for tissue engineering. This was revealed by James and his co-workers by designing a 3D PLGA via printing (Lee et al., 2008, Lee et al., 2005). He produced a structure with controllable features that can mimic tissues like villi for engineering the smooth muscles (Lee et al., 2008). At the moment, PLGA scaffold is used in the engineering of skin (Haddadi et al., 2008, Kumbar et al., 2008, Blackwood et al., 2008), liver (Li et al., 2006a, Wang et al., 2009), nerve tissue (Bhang et al., 2007, He et al.,

2009, Olson et al., 2009), bone (West et al., 2007, Yu et al., 2010, Simpson et al., 2008, Jabbarzadeh et al., 2008), cartilage (Tanaka et al., 2010, Andreas et al., 2011, Spalazzi et al., 2008), and tendon (Spalazzi et al., 2008, Moffat et al., 2008, Xie et al., 2010, Stoll et al., 2010).

2.6.4.1. Challenges and Drawbacks

Despite the fact that a lot of research have been conducted into a broad application of PHEs, there are still major challenges association with the use of some members of PHEs. A major challenge is the limited vasculation in scaffolds and tissue engineered constructs (Nerem, 2006, Meijer et al., 2007, Lovett et al., 2009, Fuchs et al., 2009). Also, PGA for example when degraded leads to the production of a significantly high amount of glycolic acid. This has been linked to strong and unwanted inflammatory response in the body (Ceonzo et al., 2006, Pihlajamäki et al., 2006, Otto et al., 2010). Also, limitations such mechanical material failure, infections associated with material and immunogenic reactions are still encountered (Patel and Fisher, 2008). Another problem confronted by engineers is the reabsorption time of some of these scaffolds. At the moment, it is still challenging to perfectly mimic all the tissues and organs in the body by using scaffolds. However, work is in progress in developing tissue engineered liver, nerve, kidney, intestine, pancreas and even heart muscle and valves. It is interesting to know that even in the midst of these challenges, significant successes have been attained in the area of skin (Yannas et al., 1989), bladder (Atala et al., 2006), airway (Macchiarini et al., 2008) and bone (Schimming and Schmelzeisen, 2004, Warnke et al., 2004), where tissue-engineered constructs have been used successfully in patients.

CONCLUSION AND FUTURE PROSPECTS

This chapter discussed the potential applications of PHEs in different areas in tissue engineering due to the excellent properties they possess. Their biocompatibility and good material properties have paved the way for a wide range of applications in tissue engineering, hence, the attention they have drawn. These inherent properties of biopolymers have given them an advantage over conventional polymers and allows for their modification. This has opened them up to new applications while at the same time enhancing them for current application. Although the challenges associated with the use of these scaffolds are vast, they still provide immensely a lot of opportunities that can help improve human health in various areas. In view of some of the limitations mentioned previously, it is important that scaffolds designed for tissue engineering applications undergo further modifications. Also, more academic attention in terms of research and study can still be done in order to develop new methods of synthesizing these biopolymers for less complexity and cheaper production cost. Without doubts, this

field holds a lot of promises in stock in the clinical and commercial arenas, especially in conjunction with new technologies and scientists from various disciplines.

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Chapter 6

POLYHYDROXYALKANOATES (PHAS) AS SCAFFOLDS FOR TISSUE ENGINEERING

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ABSTRACT

Tissue engineering is a field that has gained a lot of advancement since the discovery of biopolymers. Biopolymers are polymers produced by living organisms; that is, they are polymeric biomolecules. They consist of monomeric units that are covalently bonded to one another in order to form larger structures. Biopolymers have been widely used as biomaterials for the construction of tissue engineering scaffold. Scaffolds have been used for tissue engineering, such as: bone, cartilage, ligament, skin, vascular tissues, neural tissues, and skeletal muscles. Polyhydroxyester is a typical example of biopolymers that have been employed for this application. Their exceptional properties such as high surface-to-volume ratio, high porosity with very small pore size, biodegradation, and mechanical property have made them gain a lot of attention in this field. Also, they have advantages which are significant for tissue engineering. This chapter will focus on the production, modification, properties and medical applications of polyhydroxyesters, such as PLA (Polylactide), PGA (Polyglycolide or poly(glycolic acid)), PCL (Poly-caprolactone), poly(ester amide)s and PLGA (Poly(lactide-co-glycolide), with particular emphasis on the different polyhydroxyalkanoates (PHAs), which have diverse applications in tissue engineering.

Keywords: biopolymer, scaffolds, polyhydroxyesters, polyhydroxyalkanoates (PHAs) tissue engineering

INTRODUCTION

Without a doubt and considering the recognition and approval from the FDA and of course their well-documented biocompatible properties, biodegradable poly(α-hydroxy esters) stand out as the most widely used polymers for the fabrication of tissue engineered scaffolds and well-documented biocompatible properties. With these well-documented properties, each member of the poly(α-hydroxy esters) family has the additional option or possibility of mixing or co-polymerizing two or more of poly(α-hydroxy esters) at varying ratios in order to come up with materials with a wide array of properties, which is another interesting characteristic of the poly(α-hydroxy esters). There are at least, six poly(α-hydroxy esters) that are FDA-approved for nanofiber production. They include: poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), poly(D,L-lactic acid) (PDLLA), poly(D,L-lactic-co-glycolic acid 50:50) (PLGA5050), poly(D,L-lactic-co-glycolic acid 85:15) (PLGA8515), and poly(ε-caprolactone) (PCL). The chemical structures of some PHAs are shown in Figure 1. A number of essential considerations must be bore in mind, when using these materials in medical, food packaging and biomedical applications. These considerations, include: cytotoxicity, biocompatibility, carcinogenicity and biodegradability.

As an intracellular energy storage material, polyhydroxybutyrate (PHB) is a natural biodegradable polymer that is produced by different types of bacteria. It has numerous advantages, such as: abundant availability, biodegradability, and biocompatibility. It has physical properties that are comparable to many petroleum-based thermoplastic, such as: polypropylene and polyethylene. PHB has the potential to substitute a number of devices in biomedical and packaging fields.

The restoration of pathologically altered tissue architectures *via* the transplantation of cells in combination with appropriate supportive scaffolds and biomolecules are the main strategies of regenerative medicine. Resulting from the spectacular advances in nanotechnology, there has been increasing recognition of the potential of nanomaterials as biocompatible and biomimetic scaffolds for cells, which have provided new tools for tissue engineering and the development of 3-D cell cultures. Ordinarily, in normal or native conditions/environments, cells are embedded within and are in contact with the extracellular matrix (ECM), which acts both as a structural supporter as well as a regulator of cell activities. Essentially, tissue engineering is concerned with the use of biocompatible materials that have sufficient structural and mechanical integrities and good enough to mimic the organization of the native tissue. In order to achieve this, the scaffolds must be able to work in tandem together with isolated cells of a healthy part of the ligament of the patient itself or other cell sources, such as: stem cells and a good consideration must be given to the growth factors in order to regulate the function of these cells.

Without a doubt, there is the shortage of organs to meet the demand of organ replacement, globally. Therefore, tissue engineering that can be aided by cell transplantation may just be the route to expedite efforts. This is because, organ failures or malfunctioning often require full organ transplantations, which are most often, not always available or compatible with the patients. Consequently, tissue engineering has the potential and ability to regenerate any kind of tissue or organ in the body. In this respect, in tissue regeneration process, cells are often seeded onto a scaffold where they are allowed to grow into a new specific tissue. Even though metals, ceramics or glasses can be employed for the fabrication of the scaffolds, it is obvious that polymers, in most instances, are equally suitable candidates, hence polymers, such as: poly(L-lactide) (PGA), polyglycolide or polyglycolic acid (PGC), polycaprolactone (PLC), poly(ester amide)s (PEAs) and poly(lactide-*co*-glycolide (PLGA), have found applications as scaffolds in tissue engineering.

As a result of their excellent mechanical properties that make them to be amenable to material processing thermos-processing, biodegradable poly(ester amide)s have, of recent, been used as biomaterials because of their desirable chemical and biological characteristics [1]. In their study, Cui et al. [1] reported on the electroactive tetraaniline (TA) grafted poly(ester amide)s, which were synthesized and characterized. They concluded that the poly(ester amide)s-graft-tetraaniline copolymers (PEA-*g*-TA) synthesized, displayed excellent electroactivity, good mechanical properties and good degree of biodegradability. They further reported that the biocompatibility of the PEA-*g*-TA copolymers, systematically studied *in vitro*, demonstrated their nontoxicity with favorable adhesion and proliferation of mouse preosteoblastic MC3T3-E1 cells. It is believed that the PEA-*g*-TA copolymers synthesized can be stimulated by pulsed electrical signal which should be able to serve the promotion of the differentiation of MC3T3-E1 cells when compared with TCPs. It was therefore, concluded that the biodegradable and electroactive PEA-*g*-TA copolymers synthesized had properties that should favor the long-term potential *in vivo* application (electrical stimulation directly to the desired area) as bone repair scaffold materials in tissue engineering.

By combining the excellent thermal and mechanical properties of polyimides with the biocompatibility and biodegradability of polyesters can yield poly(ester amide)s (PEAs) of great importance Poly(ester amide)s (PEAs) are very important synthetic polymers with applications in many fields with a wide variety of applications in many fields, tissue engineering and drug delivery [2]. The combination of ester group and amide groups brings about, in a single material, immense possibilities of having biodegradable and excellent performance properties, with tunable possibilities by varying the ester:amide ratios in the material and even its hydrophilic possibilities [3]. With these possibilities

and with the synthetic polymers amiable to modifications, wide range of applications, such as: tissue engineering, controlled drug delivery systems, non-viral delivery vectors, hydrogels and smart materials are quite feasible.

Liu et al. [4] reported on the electrospinning of aniline pentamer-*graft*-gelatin/PLLA nanofibers for bone tissue engineering. In their work, they blended aniline pentamer-*graft*-gelatin (AP-*g*-GA) and poly(L-lactide) (PLLA) and electrospun the blends in order to prepare uniform nanofibers as biomimetic scaffolds. The concluded that the nanofibers exhibited good electroactivity, thermal stability and biodegradability. They evaluated the biocompatibility of the nanofibers *in vitro* by adhesion and proliferation of mouse preosteoblastic MC3T3-E1 cells. They concluded that the cellular elongation was significantly greater on electroactive AP-*g*-GA/PLLA nanofibers than on PLLA nanofibers. They also found out that the AP-*g*-GA/PLLA nanofibers stimulated by an electrical pulsed signal could promote the differentiation of MC3T3-E1 cells when compared with pure PLLA nanofibers and their results demonstrated that the biodegradable and electroactive AP-*g*-GA/PLLA nanofibers had the potential for application *in vivo* as bone repair scaffold materials in tissue engineering.

It is obvious that conducting polymers have found a variety of applications as biomaterial components as they effectively serve to deliver electrical signals from an external source to the seeded cells. Several cell types, such as: cardiomyocytes, neurons and osteoblasts respond to electrical signals by improving their functional outcomes. In this regards, Qazi and Aldo [5] reviewed the electrically responsive tissues by using polyaniline-based polymers for tissue engineering application. Polyaniline (PANI) easily comes to mind as a popular choice as an electrically responsive for tissue engineering applications, among several other polymers. This is due to its desirable properties, such as: its ease of synthesis, tunability conductivity, environmental stability and of course, its biocompatibility. In its pure form, PANI exhibits good biocompatibility, *in vitro* and *in vivo*. In addition, PANI has been combined with a host of biodegradable polymers in composites making that have attractive mechanical, electrical and surface properties. Of recent, there has been the functionalization of polyaniline oligomers with end segments that make it biodegradable and improve its biocompatibility. These (biodegradability and biocompatibility) are two properties that make these materials to be highly useful for applications in tissue engineering.

Hardy and Lee [6] reviewed the use of conducting polymer-based materials as tissue scaffold for the replacement or restoration of damaged or malfunctioning tissues, since such tissues respond appropriately to electrical stimulation. The review succinctly focused on conducting polymer-based materials with good biomimetic chemical, mechanical and topological properties and they reviewed the recent progress for the fabrication of clinically relevant tissue scaffolds.

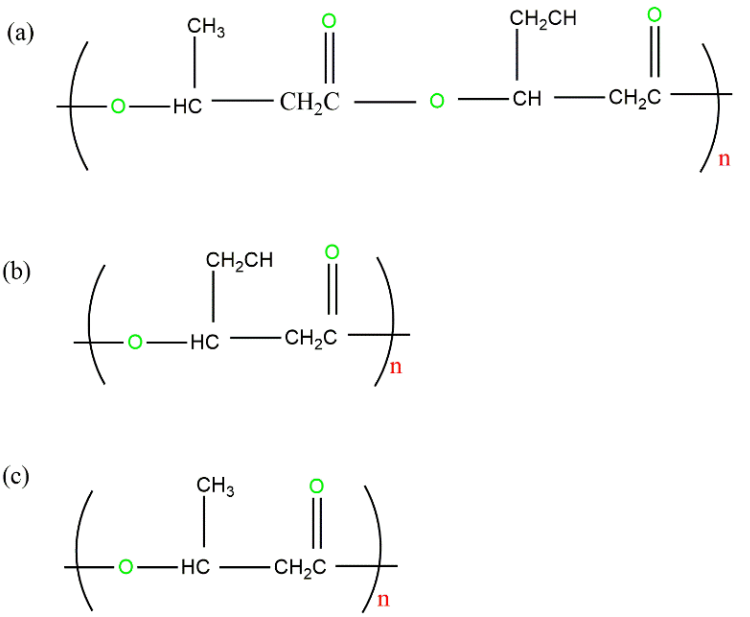

Figure 1. Chemical structures of some polyhydroxyalkanoates (PHAs): (a) PH3B, (b) PHV and (c) PHBV.

Li et al. [7] reported on the electroactive nanofibrous biomimetic scaffolds by thermally induced phase separation. In their work, they fabricated functional nanofibrous scaffolds from blends of polylactide with other functional polymers by a thermally induced phase separation (TIPS) technique. This was done *via* the fabrication of electroactive nanofibrous scaffolds from the blends of polylactide and an electroactive degradable tetraaniline–polylactide–tetraaniline (TPT) block copolymer by TIPS. This was achieved by synthesizing the TPT copolymer *via* the coupling reaction between the carboxyl-capped tetraaniline and polylactide. They further characterized the: chemical structure, electroactivity, thermal properties and mechanical properties of TPT and polylactide/TPT blend. The effect of the polymer concentration, phase separation and the aniline content on the electrospun nanofibers was investigated. They further investigated the adhesion and proliferation of C2C12 myoblast cells and protein adsorption on the electroactive biodegradable substrates. They concluded that the electroactive materials are non-toxic and that they can enhance the C2C12 cell proliferation without electrical stimulation. They also found out that the materials adsorbed more proteins when compared to polylactide. In addition, they concluded that the electrical stimulation on the electroactive substrates significantly increased the cell proliferation of C2C12 myoblasts. They are of the view that the work can opens the route for the fabrication of functional nanofibrous scaffolds from blends of polylactide and other functional polymers via the TIPS route. Ma et al. [8] reported on the electrospinning of nanofibrous electroactive scaffolds from a chitosan-grafted-aniline tetramer for tissue engineering. Tissue regeneration requires functional degradable biomimetic scaffolds. They synthesized

electroactive scaffolds from chitosan-grafted-aniline tetramer (CS–AT) by amidation reaction between the carboxyl group of aniline tetramer and the amine group of chitosan. They characterized the structure of the material by a variety of techniques, including ^{1}H NMR, FTIR, etc. The biocompatibility of the materials was evaluated by cell adhesion and proliferation of C2C12 myoblasts and dog chondrocyte cells. They concluded that the CS–AT materials had good biocompatibility and that they greatly enhanced the cell adhesion and proliferation of C2C12 cells.

Cui et al. [9] reported on the *in situ* electroactive and antioxidant supramolecular hydrogel based on cyclodextrin/copolymer inclusion for tissue engineering repair. They prepared injectable electroactive and antioxidant hydrogels by mixing tetraaniline functional copolymers and α-cyclodextrin (α-CD) aqueous solution. They concluded that UV-vis showed that the hydrogels had good electroactive properties and the antioxidant of the copolymer was ascertained. They also conducted experiments on the gelation mechanism and other properties of the system by using WAXD, DSC and rheometer. They concluded that the encapsulated cells are highly viable in the hydrogels, which suggest that the hydrogels have excellent cyto-compatibility.

Ghosal et al. [10] reported on use of poly(ester amides) (PEAs)-scaffold for tissue engineering application. They reviewed, extensively, the methods of preparation, characterization, properties and applications. They also discussed issues that relate to the electrospinning for the fabrication of scaffolds of the said scaffolds. Sun et al. [11] studied the integration of poly(3-hexylthiophene) conductive stripe patterns with 3D tubular structures for tissue engineering applications. The 3D structures contained spatially distributed conductive stripe patterns of poly(3-hexylthiophene) (P3HT) and polylactic acid (PLA), by using a confined evaporative self-assembly (CESA) technique on a flexible polyimide (PI) film. They believed that the tubular structures can possibly provide contact cues in order to guide the growth and alignment of pre-osteoblasts and smooth muscle cells. They also are of the opinion that the spatially-arranged electric signals can from conductive stripes that can regulate the proliferation and osteogenic differentiation of pre-osteoblasts. They believed that simple as this process seems, it can be effective and has the potential to mimic tubular tissues and has great promise in bone, cardiac and neural tissue engineering applications.

There is a great demand in the field of tissue engineering, which necessitates a number of PHAs to be vigorously set for investigation as biomaterials [12]. These biomaterials include, but not limited to: poly[(R)-*3*-hydroxybutyrate] [P(3HB)], poly(4-hydroxybutyrate) [P(4HB)], poly[(R)-3-hydroxybutyrate-co-4-hydroxybutyrate] [P(3HB-*co*-4HB)], poly[(R)-3-hydrox-ybutyrate-*co*-(R)-*3*-hydroxyvalerate] [P(3HB-*co*-3HV)] and poly[(R)-3-hydroxyoctanoate-*co*-(R)-*3*-hydroxyhexanoate] [P(3HO-*co*-3HHx)]. As emerging materials, PHAs and it composites, have tremendous potentials for use in medical devices, such as: cardiovascular patches, bone plates, sutures and surgical mesh. In their work, Ying et al. [13] investigated some scaffolds made from electrospun

polyhydroxyalkanoate copolymers. They studied the fabrication, characterization, bioabsorption and tissue response of these scaffolds. They studied the subcutaneous implantation of the fibers in rats' in order to investigate their bioabsorption behavior and tissue response and further studied the fibres before and after the *in vivo* experiments using different analytical techniques. They determined the structures and properties of the electrospun PHA copolymers and compared these characteristics with those of electrospun poly[(R)-3-hydroxybutyrate] and concluded that content and type of the second monomer and the diameter of fiber influenced, significantly the bioabsorption of the products and that the tissue response improved with the high content of 4-hydroxybutyrate. Amongst the numerous uses of PHAs include: the neuronal ad tissue regeneration using the piezoelectric properties of P(3HB) [14], healing support for tissues and organs, as mechanical barrier to protect organs, and nerves and tendons of a scarred tissue [15], just as the mechanical barrier of PHAs is readily available to protect organs, nerves and tendons of a scarred tissue [16] orthopedic implants, stents, vascular patches [17], tissue engineering scaffold for heart valves [18, 19] and of course, drug delivery substrate.

It is of necessity that researches in biopolymers for biomedical applications concentrate on the development of new polymers that have a well-defined metabolic route, absence of toxicity, enhancing the solubility of the drug and reducing immunologic responses [20]. As stated earlier, the focus of this chapter, will mainly be placed on polyhydroxyalkanoates (PHAs). Brief examples of applications of PHAs in the tissue engineering field will be highlighted.

SYNTHESIS/PRODUCTION OF POLYHYDROXYALKANOATES (PHAS)

For many microorganism species, polyhydroxyalkanoates (PHAs) are intracellular carbon storage mechanisms. These polymers can be synthesized *via* numerous methods. Bearing in mind that PHAs are intracellular products, the methodologies for the extraction of the polymer can be complex and expensive. Currently, solvent extraction, especially by using chloroform as a solvent, is the main route, which yields PHA with high purity, with little or no degradation of the PHA molecules. However, the potential toxicity of the solvents, cannot be ruled out. PHA synthase (PhaC) is an essential enzyme in the biosynthesis of PHA. PHA synthase (PhaC), can be classified into four groups (classes I to IV), based on the substrate specificity and the subunit composition. Detailed classification of PHA synthase was done by Rehm [20].

Khandarea and Minko [21] in their review, discussed the current synthetic advances in polymer-conjugation with different bioactive components of clinical importance. This

is even more so since it is essential to elucidate the structure activity relationship (SAR) of a drug when it is conjugated with a polymer by using different conjugation sites because this can vary the efficacy and mechanism of action when compared with its free form of drug unconjugated with any substrate (polymer, most often). It is important that the reactivity of polymer and drug must be enhanced and this is especially significant when high molecular weight linear polymers and bulkier unstable drugs, such as steroids and chemotherapeutic agents are in use. In their review, Khandarea and Minko [21] described the strategies needed to reduce steric hindrance and at the same time, increase the reactivity of the polymers, drugs and bioactive agents and they highlighted the requisite structure activity relationship in polymer–drug bioconjugates.

Bunster [22] reported that these biodegradable plastics are very promising materials suitable for the production of several medical devices, such as: stents, cardiac valves, and drug delivery systems. Bunster, discussed extensively on the uses of PHAs in medicine, which include: bone repairs, ligaments, drug delivery and wounds treatment, heart diseases, nerves and cartilages.

In his conclusion, Bunster [22] stated that PHAs can have, this type of polymer offers an alternative solution for the treatment of nerve injuries, also in iliac arteries of white rabbits, but despite the negative results, these do not exactly reflect the process of biodegradation in man. He is of the view that PHA and their derivatives demonstrated adequate prognosis that showed that they can be useful materials for medical applications, because of their biocompatibility, mechanical strength and different degradation rates, several properties adjusted by monomer structures and ratios by blending with other polymers or surface modifications.

Shabina et al. [23] reviewed the production, biocompatibility, biodegradation, physical properties and applications of eco-friendly polyhydroxyalkanoates. Different PHAs can be synthesized from co-enzyme-A-thioesters of respective hydroxyalkanoic acid and they can be degraded intracellularly for reuse and extracellularly in natural environments by other microorganisms. They are of the view that in vivo PHAs exist as amorphous mobile liquid and water-insoluble inclusions, however, the *in vitro* forms, exhibit material and mechanical properties, ranging from stiff and brittle crystalline to elastomeric and mouldable forms that are similar to many petrochemical thermoplastics. Figure 2, shows the *in vivo* and *in vitro* routes for the synthesis of PHA granules, *via* two models. The two models of PHA granule formation are described, thus: (a) the micelle model and (b) the budding model (Figure 2). The defined location of the polyester synthase and to some extent the phasin protein (that activates *Aeromonas caviae* polyhydroxyalkanoate (PHA) synthase and not *Ralstonia eutroph*a PHA synthase) on the surface of the granule are described by both models. Shabina et al. [23], strongly believe that the micelle model is obviously supported by PHA granule formation *in vitro* and in the absence of membranes.

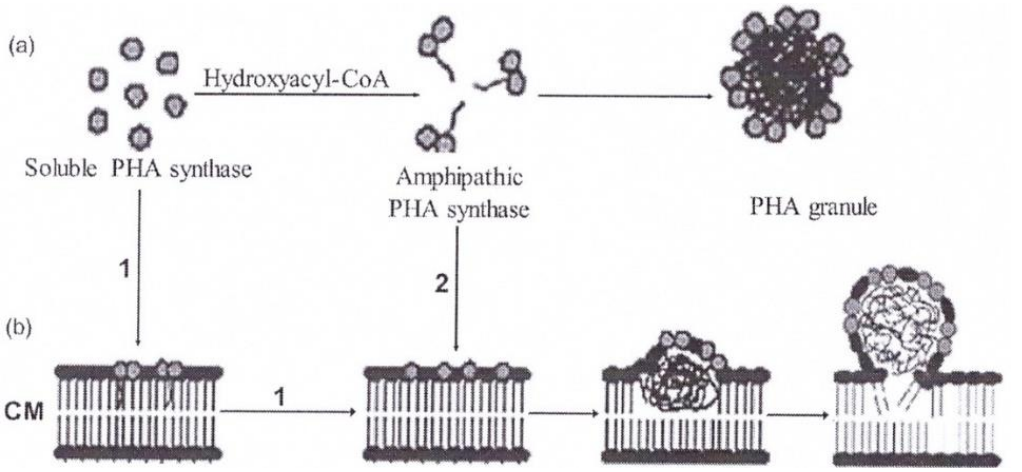

Figure 2. Models for PHA granule self-assembly by using purified polyester synthase and substrate: (a) *in vitro* assembly process, (b) *in vivo* assembly depicting two possible routes: 1 and 2. CM = cytoplasmic membrane [23].

Koller [24] recently reviewed the recent advances in PHAs production. The report dealt with individual research articles in the current global research and development landscape related to polyhydroxyalkanoates (PHA) and demonstrated how these articles are interrelated to each other. It presents a good reflection on the whole PHA process chain that includes the strain selection, metabolic and genetic considerations, feedstock evaluation, process engineering fermentation regimes and polymer processing towards high-value marketable products.

Tsuge et al. [25] reported that the amino acid substitutions at two residues downstream from the active-site histidine of polyhydroxyalkanoate (PHA) synthases are effective for changing the composition and the molecular weight of PHA. In their study, they applied the saturated mutagenesis at the position Ala505 to PHA synthase (PhaCAc) from *Aeromonas caviae* in order to investigate the effects on the composition and the molecular weight of PHA synthesized in *Ralstonia eutropha*. They varied the copolymer composition and the molecular weight of PHA by association with amino acid substitutions. They concluded that there was a strong relationship between copolymer composition and PHA synthase activity of the cells and hoped that the finding will serve as a rationale for the production of tailor-made PHAs.

Gerngross and Martin [26] reported the *in vitro* synthesis of PHB and self-assembly of spherical granules by only using purified polyester synthase and substrate. Their study clearly demonstrated that the PHA synthases possessed all the features required for self-organization into spherical particles. This was further supported by establishment of *in vitro* PHA synthesis using purified PHA synthases from other microorganisms. Peter and Rehm [27] reported that recently, fluorescence microscopic studies employing green fluorescent protein (GFP)-labelled PHA synthase, i.e., GFP was fused to the N-terminus

of class I and class II PHA synthases, without affecting PHA particle formation, thereby enabling an *in vivo* monitoring of PHA granule formation and subcellular localization.

Stubbe and Tian [28] reviewed on the role of PHA synthase on polyhydroxyalkanoate (PHA) hemeostasis. They are of the opinion that the production of PHA polymers in an economically competitive fashion *via* bioengineering requires an understanding of the biosynthetic pathway and its regulation. The review advanced the current knowledge of the mechanism of the class I and III PHA synthases, which include: the initiation, elongation and termination processes. The work also touched on the current understanding of the phase transition from soluble substrates (coenzyme-A-esters of β-hydroxyalkanoates) to the insoluble granules and their understanding of the requirement for a transcription factor, phasin proteins and depolymerases in PHA homeostasis. Han et al. [29] reported on the chemo-enzymatic synthesis of polyhydroxyalkanoate (PHA) that incorporates 2-hydroxybutyrate *via* the wild-type class I PHA synthase from *Ralstonia eutropha*. They employed a previously improved two-phase reaction system to analyze the substrate specificities and polymerization activities of polyhydroxyalkanoate (PHA) synthases. Firstly, they analyzed the substrate specificity of propionate coenzyme A (CoA) transferase and found that 2-hydroxybutyrate (2HB) was converted into its CoA derivative. Secondly, the synthesis of PHA was achieved by incorporating 2HB *via* the wild-type class I PHA synthase from *Ralstonia eutropha*. They concluded that PHA synthase stereoselectively polymerized (R)-2HB and that the maximal molar ratio of 2HB in the polymer was 9 mol%. By increasing the (R)-2HB concentration in the reaction mixture, the yields and the molecular weights of the products were decreased. They are of the view that in general, the major components of PHAs are 3-hydroxyalkanoates and was only engineered the class II PHA synthases that was reported as enzymes that have the ability to polymerize HA with the hydroxyl group at C2 position. They believed that this is the first report demonstrating that the wild-type class I PHA synthase was able to polymerize 2HB. Jurasek and Marchessault [30] employed a computer simulation approach to study the formation of polyhydroxyalkanoate (PHA) granule in *Ralstonia eutropha* cells. They suggested that a computer simulation of of polyhydroxyalkanoate (PHA) granule formation *in vivo* is able help to the design strategies in order to optimize the fermentation process and achieve high yields of PHA and can also enable the biotechnological approaches that will help to control the granule size and molecular weight of the polymer. They, therefore, developed a computer program that can simulate the formation of PHA granules inside a *Ralstonia eutropha* cell, based on published experimental data. They believed that the results are applicable to *Ralstonia eutropha* cells or other microorganisms and transgenic plants, where polyhydroxybutyrate production is made possible by heterologous expression systems. The outset of the PHA accumulation phase when the cells are small and contain no PHA granules is the start of the stimulation. In the presence of abundant glucose, the cell responds to phosphorus limitation by producing 3-hydroxybutyryl-CoA which undergoes polymerization on the

few PHA synthase molecules present in the cytoplasm. However, the cell responds to phosphorus limitation by producing 3-hydroxybutyryl-CoA which undergoes polymerization on the few PHA synthase molecules present in the cytoplasm. Then, the amphiphilic PHA synthase-PHA complex attracts additional PHA synthase molecules and granules begin to grow from these initiation sites. The limitation of phosphorus and the appearance of PHA in the cytoplasm can also stimulate production of phasin protein molecules that attach themselves to the growing granules. The continuous growing of the granules allows then to begin to touch one another and this process moves to optimize their packing. To prevent the granules from coalescing, the phasin acts as a coating agent to the granules. They concluded that the size of the cell increases and its prolate ellipsoid shape becomes closer to spherical and the accumulation process stops either when the supply of glucose is exhausted or when the granules become tightly packed within the cell, so that access to their surface is limited. Crucially are variables, such as: counts of granule-associated molecules, cell dimensions, granule size, degree of polymerization of the PHA molecules, PHA yield, etc., that are recorded in real time during the simulation. The recent advances in polyhydroxyalkanoate production by bacterial fermentation was reviewed by Lee et al. [31]. They discussed the economic production of P(3HB), by using various bacterial strains, either wild-type or recombinant and new fermentation strategies were developed for the production of P(3HB) with high concentration and productivity. In order to reduce the cost of carbon substrate, several processes for P(3HB) production from cheap carbon sources were also developed. They reported that P(3HB) can now be produced to a content of 80% of cell dry weight with a productivity >4 g/l per h. They concluded with a fermentation strategy that was developed for the efficient production of medium chain length PHA by high cell density culture. It is believed that with these advances, P(3HB) and PHAs can be produced by bacterial fermentation at a cost (~$2/kg) similar to other biodegradable polymers that are currently under development. Morais et al. [32] converted fat-containing waste from the margarine manufacturing process into bacterial polyhydroxyalkanoates. They employed a fat-containing waste produced from the margarine manufacturing process as a low cost carbon source for cultivation of different polyhydroxyalkanoates (PHAs), thus producing bacterial strains, including *Cupriavidus necator*, *Comamonas testosteroni and several Pseudomonas* strains. Essentially, the margarine waste was mainly composed of free fatty acids (76 wt.%), namely mystiric, oleic, linoleic and stearic acids. The reported that several strains were able to grow on the margarine waste, but *Cupriavidus necator r* reached the highest PHA content in the biomass of 69 wt.%. They concluded that even though the bioprocess needs to be optimized, the margarine waste did show to be a promising substrate for P(3HB) production by *Cupriavidus necator*, resulting in a polymer with physical and chemical properties similar to bacterial P(3HB) synthesized from other feedstocks. Basnett et al. [33] synthesized porous, aligned PHA fibres by using pressurized gyration process (PGP). It is a robust, cost-effective and a novel technique that generates fibres of desired

properties. They employed a PHA that is non-immunogenic, hydrophobic storage polymers produced by a variety of bacterial species under nutrient-limiting conditions. They are biodegradable in nature and exhibit thermoplastic properties [33]. This study describes the production of PHAs, synthesis of aligned porous PHA fibres using PGP and their evaluation as TES. Table 1, shows an overview of some bacterial strains for the production of medium chain length polyhydroxyalkanoate (*mcl*-PHAs) [34].

Koller [35] reviewed the current global research and development landscape of polyhydroxyalkanoates (PHAs). He discussed the interrelationships of the available that reflects the entire PHA process, chain: including strain selection, metabolic and genetic considerations, feedstock evaluation, fermentation regimes, process engineering and polymer processing towards high-value marketable products. Cavalheiro [36] reported on the heterogeneous composition of bacterial polyhydroxyalkanoate terpolymers. They produced poly(3-hydroxybutyrate-4-hydroxybutyrate-3-hydroxyvalerate) (P(3HB-4HB-3HV)) terpolymers of low 3-hydroxyvalerate (3HV) content (1.7-6.4%) with 4-hydroxybutyrate (4HB) molar fractions from 1.8% to 35.6% by fed-batch cultivation of Cupriavidus necator DSM545. Waste glycerol, γ-butyrolactone and propionic acid were used as main carbon source, 4HB and 3HV precursors, respectively. They obtained terpolymer fractions of different composition by solvent-fractionation of the original bacterial terpolymers.

They performed tensile tests on the products and concluded that the Young's modulus and tensile strength of P(3HB-4HB-3HV) decreased, whereas the elongation at break increased with the 4HB molar%, following the general trend described for poly(3-hydroxybutyrate-4-hydroxybutyrate) (P(3HB-4HB)) but with pronounced lower elasticity, while differential scanning calorimetry results indicated that the temperature of crystallization and enthalpy of melting decreased as the 4HB% increased. No crystallization was observed in terpolymers containing more than 30% of hetero-monomers (4HB and 3HV) even though multiple melting events were detected.

Table 1. Overview of bacterial strains used to produce medium chain length polyhydroxyalkanoate (mcl-PHAs) [34]

Bacterial strain(s)	**Carbon source(s)**	**Polymer(s) produced**	**Reference(s)**
Aeromonas hydrophila	Lauric acid, oleic acid	Medium chain length (mcl)-PHAs	(Lee et al. 2000, Han et al. 2004)
Pseudomonas stutzeri	Glucose, technical oleic acid, waste free fatty acids, waste free frying oil	mcl-PHAs	(Hoffmann and Rehm 2004, Fernández et al. 2005)
Pseudomonas oleovorans	Octanoic acid	mcl-PHAs	(Durner et al. 2000 Foster et al. 2005)
Pseudomonas putida	Glucose, octanoic acid, undecenoic acid	mcl-PHAs	(Tobin and O'Connor 2005 Hartmann et al. 2006)
Pseudomonas stutzeri	Glucose, soybean oil, alcohols, alkanoates	mcl-PHAs	(Xu et al. 2005)

Table 2. Some commercial polyhydroxyalkanoates (PHAs), product names, countries of origin and the producers [34]

Commercial Name	Product Name	Country of origin	Producer
Minerv-PHA	Minerv-PHA™	Italy	Bio-on
Nodax	Nodax™	USA and Japan	P&G Chemicals
Jiangsu	P(3HB)	China	Jiangsu Nantian
Tepha [35]	P(4HB)	USA	Tepha Inc.
Mirel	Mirel P4001, Mirel P4010, Mirel P2100, Mirel P4100, Mirel P2200, Mirel P5001, Mirel P5004	USA	Metabolix
Metabolix	Mvera™ B5010 Mvera™ B5011	USA	Telles LLC
GoodFellow	Polyhydroxybutrate/Polyhydroxyvalerate 12%-Biopolymer (PHB88/PHV12) Polyhydroxyalkanoates-Biopolymer (PHA)	UK	GoodFellow Cambridge Ltd.
Biocycle	Biocycle 1000, Biocycle 1189D-1, Biocycle 18BC-1, Biocycle 189C-1	Brazil	PHB Industrial
Biogreen	Biogreen	Japan	Mitshubishi Gas
Biomer	Biomer P240, Biomer P226, Biomer P209	Germany	Biomer
Ecogen	ENMAT Y1000, ENMAT Y1000P, ENMAT Y3000, ENMAT Y3000P	China	Tianan Biological Material Poly-one

PROPERTIES OF POLYHYDROXYALKANOATES (PHAS)

Been thermoplastics, PHA polymers can be moderately processed on available conventional processing equipment (injection, extrusion and compression moulders. Depending on their composition (e.g., blends and composites and copolymers), they ductile and reasonably elastic. Their properties are mostly determined by their chemical compositions, e.g., homo-or copolyester, with or without hydroxy fatty acids and the amount of such materials in the compositions. They are UV stable, in contrast to other bioplastics from polymers, such as: polylactic acid and show a low permeation of water. The crystallinity can lie in the range of a few to 70%. Processability, impact strength and flexibility improves with a higher percentage of valerate in the material. PHAs are soluble in halogenated solvents such chloroform, dichloromethane or dichloroethane [9]. However, there are several disadvantages limit that their competition with traditional synthetic plastics or their application as ideal biomaterials. These disadvantages, include: relatively their poor mechanical properties, high production cost, limited functionalities, insufficient compatibility with conventional thermal processing techniques and they can be susceptible to thermal degradation even though, PHB is slightly similar in its material properties to polypropylene (PP), has a good resistance to moisture and aroma barrier properties. Polyhydroxybutyric acid synthesized from pure PHB is relatively brittle and stiff [34]. PHB copolymers, which may include other fatty acids such as β-hydroxyvaleriate acid, may be elastic. In order to ameliorate these shortcomings, most often, PHAs require some degree of modification in order to ensure improved

performance in specific applications. Table 2, shows a list some commercial PHAs, their trade names, the producers and the countries of licencing and Table 3 shows some typical structural, mechanical and thermal properties of PHAs [37].

A few, well-established modification methods of PHAs, include:

Table 3. Some typical structural, mechanical and thermal properties of PHAs [37]

Property (units)	**Values**
WVTR: water vapour transmission rate	2.38 (g.mm/m^2.day)
OTR: oxygen transmission rate	55.12 (cc.mm/m^2.day)
Young's modulus, E	1-2 (MPa)
Tensile strength (TS), σ	15-40 (MPa)
Elongation-at-break, ε	1-15 (%)
Glass transition temperature, T_g	2 (ºC)
Melting temperature, T_m	160-175 (ºC)
Crystallinity percentage, X_{cr}	40-60 (%)

Blending of PHAs with Natural and Synthetic Polymers

- Blending PHAs with natural polymers can involve the use of: starch, cellulose derivatives and lignin
- Blending PHAs with synthetic biodegradable polymers can involve the use of: Polycaprolactone (PCL), PHA blended with PLA

Figure 3 shows a number of biodegradable polymers that can be blended with PHAs [38].

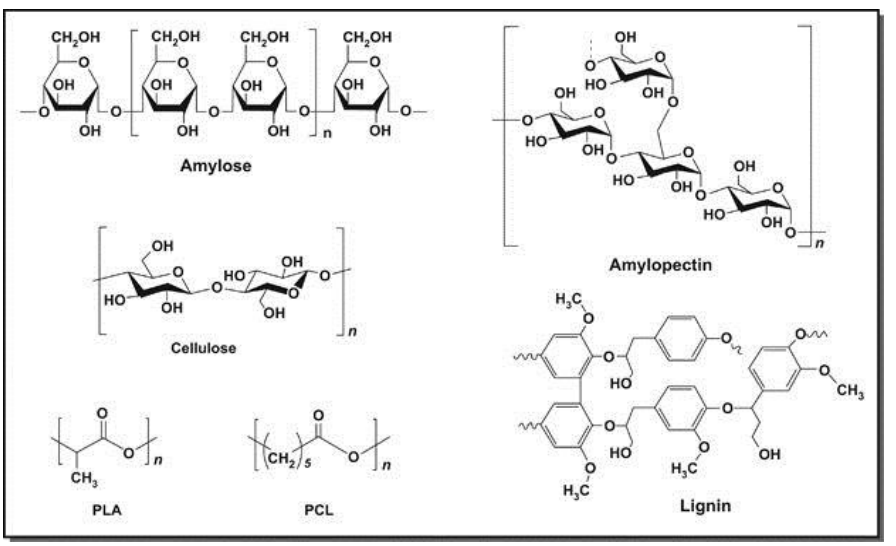

Figure 3. Suitable biodegradable polymers that be blended with PHAs [38].

CHEMICAL MODIFICATIONS OF PHAS

- PHAs modification via graft copolymerization
- PHA grafted on natural polymers

The synthesis of PHAs *via* its conjugation with natural polymers is a form of chemical modification that can improve the characteristics of PHAs, e.g., the amine group in chitosan can react with the carboxyl group-terminated PHB to yield PHB-*g*-chitosan graft copolymers, while PHBV and PHO oligomers have also been reported to graft onto chitosan to yield either PHBV-*g*-chitosan or PHO-*g*-chitosan copolymers [39-41]. Chitosan being hydrophilic chitosan will cause the PHA-grafted derivatives to exhibit an amphiphilic behavior, in which the solubility of the final product will be dependent on the degree of grafting percentage. The PHA-*g*-chitosan copolymers will have an induced controllable solubility in 2 wt% acetic acid and water can be controlled by changing the grafting percentage. The carboxyl group-terminated PHB and hydroxyl functions in cellulose generates PHB-*g*-cellulose graft copolymers through esterification reaction.

The improved properties of PHA that blends with natural raw materials or other biodegradable polymers, including starch, cellulose derivatives, lignin, poly(lactic acid), polycaprolactone and different PHA-type blends, are summarized. The functionalization of PHAs by chemical modification is described with respect to two important synthesis approaches: block copolymerization and graft copolymerization. The expanded utilization of the modified PHAs as engineering materials and the biomedical significance in different areas are also addressed.

Resulting from the reasonable advancements in the study of polyhydroxyalkanoates, a number of patents have been registered and products developed in a number of countries, as indicated in Table 2.

Other PHAs copolymers include PHA/vinyl- and (meth)acrylate-grafted copolymers and other functional PHA-grafted copolymers. Other modification techniques include: PHAs modification via block copolymerization, e.g., PHA/polyester block copolymers, PHA-polyether block copolymers, PHA-poly(methacrylate) block copolymers and other types of PHA-modified functional polymers, such as PHA-protein amphiphilic block copolymers were reported via a biosynthetic process [42]. With these modifications to PHAs, depending on the various intended usages, purposes and functions (some mentioned in the next subsection), their properties can be greatly improved for that particular purpose. A few of such usages will be highlighted, briefly below.

Use of Polyhydroxyalkanoates (PHAs) Scaffolds in Tissue Engineering

It is essential for issue engineering scaffold (TES) to have an architecture that facilitates tissue formation. They should be non-immunogenic in nature in order to prevent any inflammatory reactions and should be able to resorb at a controlled rate. In addition, its surface topography should enhance cell adhesion and proliferation. Since polymer fibres have high surface area, they have emerged as a new class of biomaterials for tissue engineering applications [43].

A number of PHAs, especially: poly 3-hydroxybutyrate (PHB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), poly 4-hydroxybutyrate (P4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) and poly 3-hydroxyoctanoate (PHO) and their composites have been used to develop devices, such as: sutures, repair devices, repair patches, slings, cardiovascular patches, orthopaedic pins, adhesion barriers, stents, guided tissue repair/regeneration devices, cardiovascular devices, tendon repair devices, bone marrow scaffolds, articular cartilage repair devices, nerve guides and wound dressings [40] and these are biomaterials suitable for use in: bone plates, bone-marrow scaffolds, joint replacements, ligaments, vascular grafts, heart valves, intraocular lenses, dental implants and medical devices like pacemakers, biosensors [43-45], skin substitutes, dural substitutes, bone graft substitutes, bone dowels, hemostats, pericardial patches, bulking and filling agents, vein valves, bone marrow scaffolds, meniscus regeneration devices, surgical mesh, ligament and tendon grafts [46].

These diverse use of PHAs, are due to the fact that polyhydroxyalkanoates can contain additives, be formed of mixtures of monomers or include pendant groups or modifications in their backbones, or can be chemically modified, all in an effort to alter the degradation rates. Depending on the compositions of the various polyhydroxyalkanoates, they can also provide favorable mechanical properties, biocompatibility and degradation times within the required desirable time frames when under specific physiological conditions.

However, pyrogenic contaminants, instead of the monomeric composition of the PHAs, sometimes co-purified with PHAs can limit their pharmacological application, therefore, it is critically essential to monitor stringently, the purity of the PHA material [45]. Resulting from the good combination with their *in vitro* biodegradation, cell and tissue compatibility, PHAs have been widely experimented for medical applications, including: medical implants applications, such as: heart valve tissue engineering, vascular tissue engineering, bone tissue engineering, cartilage tissue engineering, nerve conduit tissue engineering as well as oesophagus tissue engineering [47, 48].

Tissue Engineering Scaffolds (TESs)

These are natural polymer and synthetic polymer scaffolds and the several types of scaffolds.

Depending on its intended usage, scaffold materials can be synthetic or biological, degradable or non-degradable. Owing to their bioactive properties, natural materials have better interactions with the cells and these characteristics enable them to enhance the cells' performance in biological system. As protein, natural polymers can be classified thus: wool, silk, gelatin, fibrinogen, collagen, keratin, actin, myosin and elastin, while as polysaccharides, natural polymers can be classified, thus: cellulose, chitin, amylose and glycosaminoglycan or polynucleotides (DNA, RNA).

Chao et al. [49] studied the effects of various monomers and the micro-structure of polyhydroxyalkanoates on the behavior of endothelial progenitor cells and endothelial cells for vascular tissue engineering. This effort was in an attempt to assess the potential application of PHB and PHBV as scaffolds that can be seeded with human umbilical vein endothelial cells (HUVECs) or endothelial progenitor cells (EPCs) for vascular tissue engineering. They prepared PHA films with various surface characteristics *via* solution-casting (surface roughness) and electrospinning (mesh-like structure) techniques. Following the analysis of the mechanical and physical properties of the various types of PHA films they followed up by examining the PHAs films for cytotoxicity, biocompatibility and proliferation ability by using cell lines (3 T3 and L929) and primary cells (HUVECs and EPCs). Fluorescence microscope and scanning electron microscopy were employed to observe the cell morphology cultured on the PHA films. Furthermore, cultured EPCs on various types of PHA films were analyzed in order to establish, whether or not, the cells maintained the abilities of Ac-LDL uptake and UEA-1 lectin binding and exhibited specific gene expressions, including: VEGFR-2, vWF, CD31, CD34 and CD133. They also studied the cell retention rate and also the anti-coagulation ability of HUVECs or EPCs cultured on the various types of PHA films were also evaluated at the indicated time points. They concluded that PHA films that were prepared *via* electrospinning techniques (Ele-PHB and Ele-PHBV), had good mechanical and physical properties and that HUVECs and EPCs can attach and grow on the Ele-PHB and Ele-PHBV films, with no cytotoxicity exhibited In addition, they found out that after a week culture, expanded HUVECs or EPCs maintained their appropriate cell morphologies and exhibited adequate cell functions, such as: high cell attachment rate and anti-coagulation ability. They then concluded that the Ele-PHB and Ele-PHBV films were ideal bio-based polymers that can suitably combine with HUVECs or EPCs for vascular tissue engineering.

Cardiovascular Products

The uses of PHAs in medicine and pharmacy, have been mainly, in the cardiovascular products, which include: heart valves, atrial cardiovascular stents, vascular grafts, artery augmentation and pericardial patch **[**50]. Wu et al. [51] reviewed the many achievements on PHAs for medical devices, such as: tissue repair, artificial organ construction and drug delivery development and for nutritional/therapeutic uses. They are of the view that considering the recent FDA approval of P4HB for clinical applications, PHA applications in the medical fields is expected to have very bright future.

Hinderer et al. [52] extensively reported on the generation and assessment of functional biomaterial scaffolds for applications in cardiovascular tissue engineering and regenerative medicine. Globally, the major cause of death is as a result of cardiovascular diseases (CVDs), such as: coronary heart disease, rheumatic heart disease, congenital heart disease, myocardial infarction (MI) and strokes. The production and design of biomaterials for tissue repair, have registered significant progress. Especially, biomimetic materials that can mimic extracellular matrix (ECM) architecture and provide potentially controllable *in vivo*-like micro-environments for cells, are highly promising materials.

Patients with damaged tissues or organs may find some succour in the use of tissue-engineered scaffolds because of their biocompatibility. Yang et al. [53] reported on the use of the poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) film was irradiated by the low temperature atmospheric plasma and then coated by the silk fibroins (SF) for cardiovascular tissue engineering. Following plasma treatment, the surface of the PHBHHx film produced, became rougher and more hydrophilic than that of original film. The PHBHHx was flushed by phosphate buffer solution (PBS), shows that the coated SF exhibits stronger immobilization on the plasma-treated film than that on the untreated film. The also concluded that the cell viability assay demonstrates that SF-coated PHBHHx films and treated by the plasma, significantly supports the proliferation and growth of the human smooth muscle cells (HSMCs). They concluded that the silk fibroins modified the plasma-treated PHBHHx film, thereby providing a material that has the potential for application in the cardiovascular tissue engineering.

Taking into account, its strength and elastic properties that can be adjusted by changing its monomer contents, PHBHHx can easily be tailored to meet the requirements for regenerating both bone and soft tissues and with its with its excellent mechanical and thermal properties, PHBHHx has been used as a scaffold for tissue-engineered cardiovascular products [54].

Sutures

A biomaterial device, either natural or synthetic that can be used to ligate blood vessels and approximate tissues together, is known as a suture. As a consequence of surgery or trauma, a suture brings (and hold) together the affected tissues, just as it can be regarded as a mechanical means of wound close. Krishna et al. [55] reviewed the chemistry, production, properties, biodegradability and performance of absorbable polymeric surgical sutures. The biocompatibility of PHAs is rather good and therefore, makes it a suitable candidate for medical applications. One of its admirable properties is that it sinks in water, while PP floats and this property, facilitates its anaerobic biodegradation in sediments and of course, it is completely non-toxic. Of course, the commercialization of PHA sutures is impeded by the high cost of its production.

Shishatskaya et al. [56] implanted polyhydroxyalkanoate (PHA) sutures in order to test animals intramuscularly and their tissue reaction was investigated and compared with the reaction to silk and catgut. Two types of PHAs monofilament sutures, viz: polyhydroxybutyrate (PHB) and a copolymer of hydroxybutyrate and hydroxyvalerate (PHV) were tested for the strength necessary for the healing of muscle-fascial wounds. They concluded that the reaction of tissues to polymeric implants was similar to their reaction to silk and was less pronounced than the reaction to catgut. This was expressed in a transient post-traumatic inflammation (up to four weeks) and the formation of a fibrous capsule less than 200μm thick and after 16 weeks, the diameter reduced to between 40-60μm, in the course of reverse development. They are of the belief that macrophages and foreign-body giant cells with a high activity of acid phosphatase were actively involved in this process. In addition, they concluded that PHB and PHB/PHV sutures implanted intramuscularly for an extended period of up to one year, did not result in any acute vascular reaction at the site of implantation or any adverse events, such as suppurative inflammation, necrosis and calcification of the fibrous capsule or malignant tumor formation. Also, they observed no statistically significant differences in the tissue response to the polymer sutures of the two types, while capsules around silk and catgut sutures did not become significantly thinner [57, 58].

For instance, P4HB can be elongated by about 10 times its original length. The stretching process of a polymer induces orientation, hence the chains become oriented. This leads to strong fibers as P4HB fibers can be stronger than typical polypropylene sutures (410–460 MPa) and at least comparable in strength to commercial absorbable suture fibers like MaxonTM (540–610 MPa) and PDS IITM (450–560 MPa) sutures [59]. von Fraunhofer and Chu [59] are of the believe that what may set P4HB suture fiber apart from current absorbable synthetic fibers, is a lower Young's modulus, which will translate into improved handling and a different breaking strength retention profile upon implantation. The Young's modulus of oriented P4HB fiber (670 MPa), for example, is significantly lower than that of other monofilament sutures, such as: MaxonTM (2930

MPa), PDSIITM (1380 MPa) and BiosynTM (1000 MPa). For use in suture applications, P4HB fibers and multifilament yarns with a range of properties can be produced in order to provide varied starting points for making medical textile-based products, such as: engineering scaffolds, grafts, patches, tissue ligament, surgical meshes, dura, pericardial substitutes and slings.

Cartilage Repair Devices

Ye et al. [60] investigated investigate the potential of polyhydroxybutyrate (PHB)/poly(hydroxybutyrate-co-hydroxyhexanoate) (PHBHHx) (PHB/PHBHHx) to produce neo-cartilage, upon seeding with differentiated human adipose-derived stem cells (hASCs). They grew hASCs on a three-dimensional PHB/PHBHHx scaffold *in vitro* with or without chondrogenic media for 14 days. They concluded that that the live/dead cell viability assay showed no cytotoxicity and that GAG and the total collagen content in the differentiated cells increased significantly with *in vitro* culture time.

Following a 14-day *in vitro* culture, the differentiated cells grown on the (PHB/ PHBHHx) scaffold (differentiated cells/(PHB/PHBHHx)) were implanted into the subcutaneous layer nude mice for 12 or 24 weeks, while the non-differentiated cells/(PHB/PHBHHx) were implanted were used as the control group. They found out that differentiated cells/(PHB/PHBHHx) implants formed cartilage-like tissue after 24 weeks of implantation and was observed to be stained positive for collagen type II, safranin O and toluidine blue. They also observed a typical cartilage lacuna and there were no remnants of PHB/PHBHHx. Collagen type II was detected by Western blot at 12 and 24 weeks of implantation. In the control group, no cartilage formation was observed. They concluded that the study demonstrated that PHB/PHBHHx is a suitable candidate for cartilage tissue engineering application.

Neural Regeneration/Nerve Tissue Engineering

In clinical practice, peripheral nervous system (PNS) injuries are very common and often lead to permanent disability. Most often, PNS injury can result in partial or total loss of motor, sensory and autonomic functions conveyed by the lesioned nerves to the de-nervated segments of the body, the interruption of axons continuity, degeneration of nerve fibers distal to the lesion and eventual death of axotomized neurons [61]. Following peripheral nerve injuries, the capability of severed axons to regenerate and recover functional connections is dependent on several factors, such as: the age of the subject, the nerve trunk affected, the site and type of lesion and the distance over which axons must re-grow in order to span the injury. With this type of injury, relatively minimal successes

have been recorded with surgical methods, such as: allograft, autologous and allogenous organs or tissues including blood vessels and skeletal muscles.

Of recent, Aberg et al. [62] investigated PHB as an alternative to epineural suturing in the treatment of peripheral nerve injuries at the wrist/forearm level of the arm in a randomised, assessor-blinded clinical study. Their results demonstrated that PHB can be regarded as a safe alternative for microsurgical epineural suturing since there were no adverse events or complications, considered as product-related, were found. They concluded that the sensory recovery and parts of the manual muscle test suggested that by treating with PHB may be advantageous when compared to epineural suturing. They however added that based on the small amount of patients (twelve patients) involved in the study, the results should be confirmed by further large-scale efficacy studies.

For possible use in soft tissue repairs, augmentation, and bulking applications, low molecular weight oligomers of P4HB have been prepared in solution, via the hydrolysis of higher molecular weight P4HB and along with P4HB micro-dispersions could potentially be administered by injection [63].

PHAs in Orthopaedic Pins and Devices

They form as core-and-shell biopolymers or copolymers so the created bioplastics can be easily tweaked by controlling the creation of polymer layers in order to tailor the plastics for specific applications. This tailoring of plastic properties, the biopolymers' small sizes and PHAs biodegradable and biocompatible nature, make these bioplastics especially useful in orthopedic devices, orthopedic implants and other implantable medical devices.

Plastic polyhydroxyalkanoate (PHA) microstructures can be formed in bacterial hosts and have applications in orthopedic devices, orthopedic implants and other implantable medical devices. These materials are biocompatible and can degrade after being implanted. The advantages of polyhydroxyalkanoates (PHAs) for use in orthopedic devices, include:

- Polyhydroxyalkanoate (PHA) biopolymers are created in bacterial hosts
- Biopolymers are water resistant and stable at normal conditions and are easily stored
- Copolymer layers can be tailored to fit application
- Especially applicable in orthopedic devices, orthopedic implants and other implantable medical devices

Savvidis et al. [64] reviewed the use of bioabsorbable materials in orthopaedic Surgery and its association with infections. They reported that use of bioabsorbable materials is common in medicine and a number of reports have suggested the use of these implants for treating fractures and other orthopaedic conditions may lead to less implant morbidity, since they have additional advantages, such as: radiolucency, elimination of hardware removal procedures limit of stress-shielding and they gradually transfer load to healing fractures. Even though they are popular, reports of complications abound in the literature. These complications have rarely adverse effect on the long term outcome, it is believed that they are quite frequent and have been reported with most of the commercially available implants with varying incidence rates and reactions to them.

Biomedical Applications of Polyhydroxyalkanoates (PHAs)

With properties similar to synthetic polymers, PHAs are desirable materials for biomedical applications. Among the desirable properties of PHAs for use in the biomedical fields, include: biodegradability, biocompatibility and non-toxicity. These properties make them prime choice as: biocontrol agents, drug carriers, biodegradable implants, tissue engineering, memory enhancers and anticancer agents [65]. Canadas et al. [66] conducted an integrated investigation which showed that waste glycerol can be bio-valorized *via* the fabrication of electrospun scaffolds for stem cells. They reported that human mesenchymal stem cells (hMSC) can provide an interesting model of regenerating cells because of their ability to differentiate into osteo-, chrondro-, adipo- and myogenic lineages. They concluded that hMSC have modulatory properties that possess the potential to assuage the treatment of immunologic diseases. They employed biorefinery approach, by using the crude glycerol directly recovered from a biodiesel post-reaction stream, fed as major carbon source to Cupriavidus necator DSM 545 to produce polyhydroxyalkanoates at polymer titers of 9-25g/L. They produced two P(3HB-4HB-3HV) terpolymers products, one containing 11.4% 4HB and 3.5% 3HV and the other containing 35.6% 4HB and 3.4% 3HV, which were electrospun into fibers of average diameters of 600 and 1400nm, respectively.

They are of the opinion that electrospun fiber meshes offer tunable mechanical and physical properties that can mimic the structure of the native extracellular matrix, the natural environment where cells inhabit. They concluded that hMSC have modulatory properties that possess the potential to assuage the treatment of immunologic diseases. They thereafter, cultured hMSC for 7 days in both fiber meshes and concluded that both meshes showed ability to support stem cell growth at acceptable proliferation levels and that comparative results clearly demonstrated that scaffold topology is critical, with electrospun PHA fibers succeeding on the support of significant cell adhesion and proliferation, where planar PHA films failed.

Challenges and Future Trends

As commodity plastics PHAs applications may be relatively limited because of the high production cost, relatively difficulty of extraction and yet low yield, *in vivo* degradation, complexity of technology. Therefore, this main challenge in the production cost of PHAs when compared to petroleum-based plastics is definitely a challenge. However, researches have advanced and there are, now available, many routes of making the process cheaper than previously employed methods. The use of suitable bacterial strains and inexpensive carbon sources is a way forward to reducing cost. This is because the choice of the carbon source, which as nutrient can be limiting factor that can significantly affect the production rate, quality and type of polymer.

A typical cause of inflammation can be the usage resulting from the implantation of engineered biomaterials. This is as a result of the immune response of the host that may require the use of anti-inflammatory agents, e.g., steroids (glucocorticoids) or nonsteroidal compounds. Of significance, is the fact that glucocorticoids can often show strong inhibitory effects in inflammation relating to cytokines, thereby down regulating the transcription of interleukin (IL)-1, tumor necrosis factor (TNF)-alpha and IL-3, 4, 5, 6 and 8. Without a doubt, considering the wide and ever increasing versatility of PHA polymers, their applications in the medical field are simply enormous and therefore the need for PHA devices will certainly keep increasing, just as researches and invocations on this “magical” class of polyesters, continuously keep emerging.

CONCLUSION

Obviously, the delivery of bioactive compounds, especially drugs by matrices made of PHAs will have continual growing interests. This is because of the unique and convenient characteristics of PHAs drug delivery systems, with their very interesting and highly unique routes of release control. The delivery of growth factors or immune-modulators by employing PHA systems is highly envisaged. PHAs and their derivatives demonstrate usefulness as suitable materials for medical applications, because of their various characteristics, such as: biocompatibility, mechanical strength and different degradation rates and their numerous properties that can be adjusted by, e.g., types of monomer structures and ratios by blending with other polymers or surface modifications. Depending on the particular applications, stringent studies must be performed in order to examine the degradation behaviour of each type of application and the types of effects that can result in any specific conditions. For example, as per their applications in tissues engineering, researches must be focused on promoting tissue regeneration that can be easily accompanied by an appropriate degradation rate, based on tissue regeneration speed. By developing more efficient fermentation routes, recombinant organisms and

recovery processes research efforts are primarily devoted to lowering the cost of production and possibly meeting the pricing target. It is of paramount importance to explore ways of producing relatively cheaper PHAs that display excellent qualities and in the desirable quantities for mass production. In this regards, it is of paramount importance to have a good selection and development of bacterial strains that will be capable of efficient consumption and transformation of the various substrates (the required biomass) into a wind range of PHAs that possess the different properties and qualities needed for medical applications and at high yield and decent productivity routes that will give high performance fermentations and efficient extraction and purification to lower the price. It is envisaged that by using local and cheap substrates, e.g., agricultural waste, the cultivation processes that combine batch and fed-batch fermentations is expected to give the significant productivity when compared to the other reported methods.

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Chapter 7

BLENDS AND COMPOSITES OF POLYHYDROXYALKANOATES AND THEIR APPLICATIONS

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ABSTRACT

There has been a growing interest in the replacement of fossil-based polymers with biodegradable polymers, especially those produced through natural resources. Among these biodegradable polymers, polyhydroxyalkanoates with their unique properties, e.g.,

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high molecular weight (similar to conventional polymers), qualify them to be promising candidates for various industrial applications. Their high cost and their relatively low mechanical properties, limit their applications. Blending and the incorporation of various fillers, have been studied for the past decades in order to improve and/or overcome these drawbacks. In this chapter, recent studies, based on the blends and nanocomposites of polyhydroxyalkanoates in order to enhance its applicability in various fields, are reviewed. The potential applications, challenges and opportunities relating to polyhydroxyalkanoates blends and their nanocomposites, are also addressed.

Keywords: polyhydroxyalkanoates, blends, composites, nanocomposites, applications

1. INTRODUCTION

Plastics features unique properties, such as lightweight and good mechanical properties and they are easily mouldable to wide range of products, which enhance the quality of our lives. At least, more 40% of these conventional petroleum-based plastics, are used in single use disposable products, such as: plastic bags, sanitary cups and utensils. Due to their non-biodegradable nature, these plastics accumulate in landfills and in and around our natural habitats. This is evident by amount of plastic debris found in oceans and open fields, creating physical hazards for wildlife and public nuisance. In recent years, there has been growing interest in the development of plastic products that are compatible with the environment, due to the strict environmental regulations by governments, environmentalists and stakeholders, in general. Biopolymers from renewable resources, such as: polyhydroxyalkanoates (PHAs), poly (lactic acid) (PLA), thermoplastic starch (TPS), protein plastics (e.g., soy protein plastic) and cellulose derivatives, are highly and widely studied renewable polymers, as possible replacement of the traditional petroleum-based plastics for various customer products. Among these biopolymers, PHAs have received considerable amount of popularity due to their properties that are similar to the commonly used synthetic polymer, i.e., polypropylene. PHA has similar glass transition temperature, melting temperature, degree of crystallinity, Young's modulus to polypropylene, which afford its use in different applications [1]. There are, however, some disadvantages associated with PHA, such as: low melt elasticity, poor thermal stability, brittleness, low crystallization rate and high cost, which impede its success. Therefore, its success depends on overcoming these shortcomings. Blending PHAs with other polymeric materials as well as the incorporation of different nanofillers, serve as most effective and cheaper route(s) to overcome their shortcomings. In this chapter, the recent developments on the blending and the addition of the nanofillers to PHA in order to overcome its limitations for various applications, e.g., biomedical and packaging, are discussed.

2. STRUCTURE AND PROPERTIES

PHAs are a family of polyester with a structure illustrated in Figure 1. It is synthesized by various archea and bacteria (e.g., *Cupriavidus necator, Alcaligenes latus, Aeromonas hydrophila, Pseudomonas oleovorans, Pseudomona aeruginosa* and *Haloferax mediterranei*) with more than 150 monomer compositions that yield polymers with different properties and applications [2]. They are produced in nature by these microbes under stress conditions i.e., high carbon availability and limitations of other nutrients, such as nitrogen, potassium, phosphorus, magnesium and oxygen [3]. In such a stress condition, i.e., high carbon: nitrogen (or potassium, oxygen, magnesium, phosphorus) ratio, microbes manipulate their metabolic activities in such a way that rather than following the tricarboxylic acid cycle, the acetyl-CoA is diverted towards the synthesis of polyhydroxyalkanoates (PHAs). A wide range of PHAs containing 3-hydroxybutyrate (3HB) and a second monomer unit, such as: 4-hydroxybutyrate (4HB), 5-hydroxyvalerate (5HV), 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx) or 3-hydroxy-10-undecanoate (3HU), have been produced by utilizing different carbon sources, culturing conditions and bacterial strains. Metabolic engineering can also be used to combine the various monomers with different properties. In Figure 1, the substituent R can be CH_3 for polyhydroxybutyrate (PHB), C_2H_5 for polyhydroxyvalerate (PHV), C_3H_7 for polyhydroxyhexanoate (PHH) and C_4H_9 for polyhydroxyoctanoate (PHO) and many others. PHA is categorized into two groups, based on the number of carbon in the monomer units, i.e., small chain length *(scl)* PHA, when the monomer units contain 3 to 5 carbon atoms and medium length chain *(mcl)* PHA, when the monomer has 6 to 14 carbons. The commercially available PHAs, include: polyhydroxybutyrate (PHB), poly (3-hydroxybutyrate-co-3hydroxyvalerate) (PHBV) and poly (3-hydroxybutyrate-*co*-4-hydroxyvalerate) (PHBH). It can clearly be seen that both the latter polymers are copolymers, composed of 3-hyrxoxybutyrate units and a small amount of medium chain length 3-hydroxyalkanoates *co*-monomers with side groups having at least 3 carbon units. This has been one of the processes to improve the properties of PHAs, i.e., different copolymers at varying ratios in the polymer chain (Table 1). Other method includes the elaboration of PHA in order to prepare multiphase systems, *viz*. incorporation of different fillers and/or other polymers. The most researched copolymer in the PHAs family, is PHBV. This is due to its properties that are comparable to PP, with regards to: crystallinity, melting points and glass transition temperature. One of the major drawbacks of PHAs is the upscaling of production. Moreover, physical and chemical properties, such as: reducing melting temperature (which is close to its degradation temperature) and glass transition temperature, elastic modulus, tensile strength and elongation, are dependent on the monomeric units and the molecular weight of the polymer. The combination of these units in order to come up with copolymers with high molecular weight, can overcome some of these limitations. For example, Poly-3-hydroxybutyrate

(P3HB) has high crystallinity of ~70% and high modulus (~3.5 GPa) and the addition of 3-hydroxyvalerate (3HV) comonomer yields the copolymer P(3HB-*co*-HV) that has less crystallinity and modulus (~1.5GPa) with improved elongation-at-break [4]. On the other hand, P(3HB-*co*-4HB) features less crystallinity, which can further be reduced from 70% to ~14%, by increasing 4HB from 0 to 49 mol%. By playing around with these co-monomers, it is possible to tune the mechanical properties and processability of the resulting PHA copolymer. Furthermore, the manipulation of the production, such as feed composition, culturing conditions, increased microbial biomass, high expression of polymerase genes and genetic modifications to synchronizing the termination of PHA synthesis with cell lysis, can enhance the production. Overall, PHAs are biodegradable polymers with their biological (microbial) origin and their non-toxic nature which afford their applications in the biomedical and packaging fields. Moreover, their compatibility with the environment and their comparable properties to the available synthetic polymers, permit their application in other sectors.

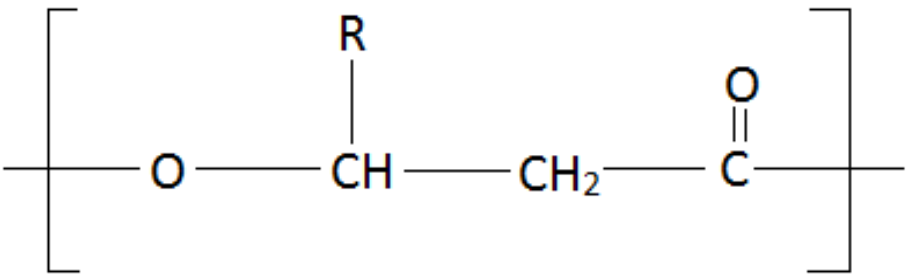

Figure 1. Schematic presentation of general structure of polyhydroxyalkanoates (PHAs).

3. PHA BLENDS

Modifications to polymers, overtime, have been beneficial in improving the properties that are deficient, as identified by many researchers. Improvements to resistance to temperature, tensile strength, mechanical properties can eventually lead to a better final polymer and also utilization of certain less expensive materials and can result in lowering the cost of the eventual polymer formed. These modifications have also been employed to PHAs and are sometimes referred to as PHA blending. This blending is usually less expensive than the chemical modifications or *via* copolymer synthesis. The properties of the PHA blends can be simply altered by varying the blend composition, changing molecular masses, changing the polymers or co-polymers individually or modification reactions of the PHAs [6-8].

Table 1. Physical properties of PHAs [5]

Properties	**Poly (3-HB)**	**Poly (3HB-*co*-3-3% HV)**	**Poly (3HB-*co*-3-11% HV)**	**Poly (3HB-*co*-3-20% HV)**	**Poly (3HB-*co* 4-3% HB)**	**Poly (HB-*co*-4-16% H)**	**Poly (3HB-*co*-4-64% HB**	**Poly (4-HB)**	**Poly (3HB-*co* 3HV-*co*-4HB)**	**Poly (3HB-*co*-3HV-*co*-3HHx)**	**Poly (3HO-*co*-3-12% 3HH)**
Melting temperature, T_m (°C)	177	170	157	145	166	152	50	60	140	114	61
Glass transition temperature, T_g (°C)	4	-	2	-1	-	-8	-	-50	-2	-2	-35
Tensile strength (MPa)	40	38	38	32	28	26	17	104	-	8	9
% elongation-at-break	6	-	5	50	45	444	591	1000	-	481	380

The blending of PHAs has been understudied by numerous researchers with materials from biopolymers to the use of rubbers. Gerard and Budtova [7] investigated the blending of PHA and PLA and reported on their immiscibility. They also investigated the blending of PLA and PHBV, reporting that although both polymers are both brittle, blending little quantities of PHBV with PLA revealed plastic deformation which was significantly ductile. In another report, Noda and colleagues [9] showed that blending PHA(90%)/ PLA(10%) led to improvements in the percentage elongation of the blend, which was caused an increased the amorphous phase of the blend.

4. PHA BLENDS AND THEIR PROPERTIES

As highlighted earlier, blending of PHA leads to the alteration in the properties of the original polymers and new properties of the resulting blends emerge, i.e., thermal, mechanical, etc. that tend to differ from the original materials and this section dwells on findings reported by diverse researchers on the properties of the resulting blends in comparison to the previous materials.

4.1. Mechanical Properties

The mechanical properties of blends of PHA differ according to the individual properties of the blended materials. Overtime, researchers have reported on the mechanical properties of different PHA blends and different effects of different ratios of blending have been reported. Lourerio and his colleagues [10] in Portugal reported on the mechanical characteristics of PHA/PLA blends. Their major aim with investigating the range of blend compositions was to identify the blend composition which provided the best adhesion between the two material phases of PLA and PHA that were blended. They reported that the best synergistic blending effect resulted when PLA was the matrix and PHA the dispersed phase in a PHA/PLA (30/70), which was followed by PHA/PLA (70/30), the inverse composition [10].

4.2. Thermal Properties

Blending and modification also affect the thermal properties of the final blended product and depending on the individually materials blended, the thermal properties may be improved or worsened. The miscibility of polymers can be derived from identification of the glass transition temperature (T_g). Researchers [11] have reported the thermal

properties, amongst other properties, of blends of PHA/PLA, prepared in diverse compositions, ranging from 0–100%, differing in steps of 10%.

5. PHA-NANOCOMPOSITES

The fabrication of nanocomposites serves as one of the most effective method to enhance the properties of polymers. Nanocomposites are composed of a filler on nanoscale (discontinuous phase) embedded in a polymer matrix (continuous phase). Nanocomposites display excellent enhancements on the resulting properties, such as: mechanical, thermal and barrier properties when compared to neat polymer and/or conventional composites/blends. A wide range of nanofillers, such as: cellulose nanofibrils, carbon nanotubes and organo-modified montmorillonite, have been incorporated into PHAs in order to improve their properties. In this chapter, the thermal and mechanical properties of PHA nanocomposites, will be discussed.

5.1. Mechanical Properties of PHA Nanocomposites

PHAs have poor mechanical properties, e.g., very brittle, which impede their success in various applications. Incorporation of different nanofillers is of essence in order to improve and/or overcome these limitations. Bionanocomposites of modified and unmodified layered silicate and PHAs have been studied by numerous researchers [12, 13], as summarized in Table 2. The main objective of these studies revolved around finding good dispersion of the nanofillers in order to improve, not only the resulting mechanical properties, but also overall properties of the resulting nanocomposite products. The modification of the layered silicate and the preparation method play a significant role on their dispersion. Bionanocomposites of poly(hydroxybutyrate-*co*-cohydroxyvalerate) (PHB/HV)/orgnoclay (cloisite30B, a monotallow bis-hydroxyethyl ammonium-modified montmorillonite), were successfully prepared by melt intercalation [13]. It was found that nanoclay was well-distributed in the matrix due to the formation of hydrogen bonds with the hydroxyl groups in the organic modifier, which in turn improved the mechanical properties of the nanocomposite. The Young's modulus of the resulting nanocomposite, increased significantly with only 2wt% of clay from 480 MPa to 730MPa, while the tensile strength increased marginally, from 31 MPa to 35 MPa. The elongation-at-break was not significantly changed (from 8.5 to 7.7%). Polyhydroxybutyrate/PGV montmorillonite modified with neopemtyl(diallyl)oxy tri(dioctyl) pyrophophato titanate was prepared by using a micro twin-screw extruder, followed by extrusion in order to obtain the desired specimen samples for analysis. In this study, PHB was blended with epoxidized natural rubber (ENR) and maleate polybutadiene was used

as compatibilizer. It was reported that PHB-ENR compatibilized samples with 5wt% modified clay, showed ~400% improvement in impact properties and only 40% reduction in modulus when compared to the neat PHB. Solution casting technique can also be used to prepare the PHAs/clay nanocomposites [14, 15]. In the work of Bruzaud and Bourmaud [14], poly (hydroxybutyrate-*co*-3-hydroxybutyrate) (PHBV)-based nanocomposites with different amounts of organophilic (Cloisite 15A (montmorillonite modified with dimethyldihydrogenated tallow with ~65% C18, 30% C16, 5% C14; CEC=125 mequiv./100g), were successfully prepared through solution intercalation method by using chloroform as a solvent. Incorporation of clay increased the tensile modulus from 633 MPa (for neat PHBV) to 1677 MPa at 5wt% of filler-filled PHBV, while the tensile increased from ~6 MPa to 29 MPa. This was attributed to the high stiffness of the filler, their partial exfoliation and strong interaction with the polymeric matrix. Comparison between two commercial montmorillonites (5%m/m) (MMT), Cloisite Na^+ (Na-M) and Cloisite 30B, modified with quaternary ammonium CEC 0.90 equiv./g (30B-M) as reinforcement in polyhydroxybutyrate was studied by Botana et al. [16]. The tensile modulus and strength did not significantly increase for both clays, but a high reinforcement character was recorded for C30B, which was attributed to a better particle exfoliation/intercalation. A comparison between C30B and tubular-like clay, halloysite (HNT) was also conducted by Carli et al. [17]. Generally, the tensile modulus increased with increase in fillers content, however, the stiffness was more significant for the C30B (103%) than HNT (63%) when compared to pure matrix. It was pointed out that the high stiffness for C30B-based nanocomposites may have compromised other mechanical properties, such as: strain-at-break, tensile strength and impact strength, while HNT improved the strain-at-break, tensile and impact strengths, while maintaining high stiffness. These results indicate that it is possible to choose from different layered silicates (whether modified or not) to improve the mechanical properties of the PHAs and the choice is directly depended on the specific mechanical properties that need to be improved. Carbon nanotubes (CNTs) were also reported as potential reinforcement for PHAs [18-20]. In a recent study by Vidhate, Innocentini and D'Souza [18], multiwalled carbon nanotubes (MWCNT)/poly(3-hydroxybutyrate-*co*-3-hydroxy-valerate) nanocomposites were successfully prepared by melt blending by using a Brabender batch mixer. The mechanical properties were studied by using different techniques, such as: tensile and compression tests as well as dynamic mechanical thermal analysis. The incorporation of MWCNTs led to an increase in the tensile modulus with an increase in MWCNTs content, while the yield stress value increased for the 1 and 2wt% MWCNT compositions because of agglomeration at higher contents. In the case of compression test, the upper yield strength and modulus increased with an increase in MWCNT content, with the 10wt% inclusion, showing superior properties. The compressive strength, modulus and strain-at-break increased from 45 MPa, 250 MPa and 42% to 118 MPa, 60 MPa and 65%, respectively for the 10% MWCNT composition when compared

to neat polymer matrix. Dynamic mechanical analysis, however, showed that the storage modulus for the 10wt% composition was lower than the neat PHBV and other nanocomposites because of some agglomerations in this sample as opposed to other samples below the 10wt%, where the storage modulus increased with an increase in MWCNTs content. Modified MWCNT-based nanocomposites were prepared by using solution casting method by Ma et al. [20]. Modification of MWCNT was carried out by suspending them in H_2SO_4:HNO_3 (mixture with a volume ratio of 3:1), which was refluxed in a water bath at 80°C for 3 hours. It was reported that the 1wt% of modified MWCNT, was the maximum inclusion content to improve the overall mechanical properties (elastic modulus, tensile strength and elongations to fracture) of the nanocomposite material. The effect of silica on the properties of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) was reported by Han et al. [21]. By using the Haake batch intensive mixer, various contents of silica (1-10 wt %) were used to prepare the nanocomposites. As expected, the tensile modulus was found to increase with the amount of silica in the composition, which led to a significant decrease in elongation-at-break, since the material become stiffer. The tensile strength values obtained were found to be higher than the theoretically calculated values by using the Nicholais-Narkis model. This was attributed to the gradual increase of adhesion between the filler and the matrix with increase in the filler content, hence there was good stress transfer between the nanocomposite components.

Nanowhiskers from natural origin have received some degree of popularity due to their unique properties, such as: abundant availability, renewability and excellent mechanical properties. Numerous researchers [22-24] have used this material to reinforce PHAs in order to produce cheaper bionanocomposites. Chitin nanowhiskers can interact with PHAs matrices *via* hydrogen bonding, hence it was reported to improve the compressive modulus of PHA/chitin nanowhiskers [22]. Elsewhere, it was reported that the addition of 5wt% of chitin whiskers to poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) resulted in the tensile strength and Young's modulus to have improved by 24% and 43%, respectively, however acetylation treatment further improved these figures by 44% and 67% due to better dispersion [23]. Nanofibrilliated cellulose/poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) biodegradable nanocomposites were fabricated by melt compounding by Srithep et al. [25], by using thermokinetic mixer. Nanofibrilliated cellulose with high stiffness significantly increased the Young's modulus, while the tensile strength was found to be the same as the neat polymer. Unidirectionally aligned cellulose nano-whiskers-based nanocomposites were produced by applying electric field, during a casting process [26]. It was reported that the storage modulus decreased when the orientation angle (θ) increased from 0 to 90° due to fiber orientation. It was found that the storage modulus for the random oriented fibres sample was 35% lower than that of the corresponding 'aligned' sample, due to the reduction of agglomeration of CNWs. This is because the electric field interfered with the interactions between CNWs *via* hydrogen

bonding and therefore hindered agglomeration, which in turn improved the interfacial interaction between the filler and the matrix. A much higher content of cellulose nanowhiskers was incorporated into poly(3-hydroxybutyrate-*co*-hydroxyvalerate) by Yu et al. [27] in order to improve the mechanical properties of the ensuing nanocomposite product. The nanocomposites were manufactured *via* the solution casting method by using chloroform as a primary solvent. With a 10wt% of CNWs inclusion, the tensile strength increased by 149%, while the Young's modulus increased by 250%. Comparison between the reinforcing effect of cellulose nanowhiskers extracted *via* sulphuric (CNW-S) and hydrochloric (CNW-H) acid was investigated by Yu et al. [28]. It is recognized that both of these methods lead to different functional groups on the surface of the CNWs. The maximum tensile strength and the Young's modulus for the CNW-S (10 wt%) were improved by 149% and 250%, respectively, while for the CNW-H (10wt%), the tensile strength and Young's modulus increased, respectively by 166% and 280% when compared to the neat matrix. The difference was ascribed to the large aspect ratio, high degree of crystallinity and strong nucleation effect on the PHBV during crystallization, under a reasonably good dispersion of CNW-H in the matrix. Moreover, the large amount of the hydroxyl groups on the CNW-H surfaces, instead of sulphate groups, are beneficial to form hydrogen bonding between the filler and the matrix. It was also indicated that it is possible to increase the content of CNW-H to 12wt%, which further increased the tensile strength and the tensile modulus by 175% and 300%, while elongation-at-break reduced to 6.8%. Bionanocomposites blend of PLA and PHB produced by electrospinning technique was recently reported by Arrieta et al. [29]. In this case, acetyl(tributyl citrate) (ATBC) was used as plasticizer. The addition of only 1wt% cellulose nanowhiskers as nanofillers, increased tensile strength and Young's modulus from 4.5 MPa and 70 MPa and 16 MPa and 230 MPa, respectively.

5.2. Thermal Properties

Several reports have stated that the thermal properties are dependent on various parameters, such as: the filler-type, content of filler, treatment and/or compatibilizer used. In the case of the nanocomposites, the three degradation steps are often reported. The first and second steps ranging between 50 and 350°C, are associated with moisture evaporation, while the third degradation step, in the range of between 350-650°C, represents the structural dihydroxylation of the nanocomposite sample [32]. All these scenarios depend on the filler-type and the compatibilizer used. In [32], it was reported that PHBV/unmodified hallosite (HNT) nanocomposite displayed three degradation steps. The first and second mass losses were in the range 50-350°C, which corresponds to a loss of adsorbed water from the surface and internal channels of the tubes.

Table 2. Selected studies on the mechanical properties of PHAs nanocomposites

Sample	Preparation method	Comments	Refs.
Poly(hydroxybutyrate)(PHB)/montmorillonite (MMT)(CEC110 mequiv/100g) ion exchanged with dimethyl-ocadecylamine	Melt extrusion	Storage modulus increased with increase in clay content	[30]
Poly(hydroxybutyrate)(PHB)/synthetic fluromica (CEC 120mequiv/100g ion exchanged with dimethyl ditallow ammonium (MAE)	Melt extrusion	Storage modulus was higher than MMT-based nanocomposites due to MMT contributing to the degradation in the molecular weight	[30]
Poly(hydroxybutyrate)/commercial orgomodified MMT(Cloisite 10A)	Solution casting	Maximum improvement was achieved for 3wt% clay loaded samples due to better dispersion	[15]
Poly(hydroxyl-butyrate-*co*-hydroxyvalerate) commercial orgomodified MMT(Cloisite 10A)	Solution casting	Maximum improvement was achieved for 3wt% clay loaded samples due to better dispersion	[15]
Poly(3-hydroxyxybutyrate-*co*-3-hydroxyvalerate)(PHBV)/poly(butylene adipate-*co*-terephthalate) (PBAT)/recycled silane treated wood/Cloisite 30B	Kinetic mixer followed hot-pressing	No significant changes in mechanical properties of the nanocomposites as compared to blend composites reinforced with recycled wood, however, the presence of clay enhanced thermal stability	[31]
Poly(3-hydroxybutyrate)/single-walled carbon nanotubes and multi-walled carbon nanotubes	Solution casting	Storage and loss modulus increased by addition of carbon nanotubes (viz. from 550MPa, 10 MPa (for neat polymer) to 1250 MPa and 140 MPa for SWCNT and 1020 MPa and 120 MPa for MWCNT nanocomposites	[19]
Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)/cellulose nanowhiskers (CNW)	Solution casting	The mechanical properties (tensile modulus (77%(by tensile test) and 91%(bulge test), tensile strength (35.5%) and toughness (41%)) were improved by addition of 5wt% of CNWs as compared to pure matrix Tan delta peak shifted to higher temperatures (from 23.8 to 35.2°C for 5wt% CNW nanocmposite) and the area was reduced, while storage modulus increased with CNW content because of strong interaction between the nanocomposite components retraining polymer chains movements	[24]
poly(hydroxybutyrate-*co*- hydroxyvalerate)/hallosite nanotube (HNT)	Melt compounding	The toughness increased while maintaining high modulus for HNT treated with (3-glycidyloxypropyl) triemethoxysilane (GOPTMS) and (octyltriethoxysilane) (OTES)	[32]

The third mass loss between 350-650°C, was associated with the structural dihydroxylation of the Al-OH and Si-OH groups of HNT. However, the modification of HNT by using silanes coupling agents, *viz*: (3-aminopropyl)triethoxysilane, [3-(methylamino)propyl] trimethoxysilane, (3-glycidyloxypropyl) trimetho-xysilane and octyltriethoxysilane), resulted in the first mass loss in the temperature range of between 25-250°C, due to the organophilicity of the HNT because of the reduced water adsorbed to the HNT due to the presence of organosilanes. The second mass in the temperature range of between 200-350°C, was attributed to the decomposition of silane bonded to the clay by the secondary interactions or grafted onto Al-OH and Si-OH groups on the HNT surface, edges and interchannel, as well as the decomposition of the oligomerized silane network that was not removed during washing, the last degradation step in the temperature range of between 350-650°C, was associated with the additional organic decomposition of the silane grafted onto the HNT. The thermal stability of PHAs was also found to increase with the addition of the nanofillers. This was attributed to the high thermal resistance of the nanofillers when compared to the neat polymeric matrix. Crosslinking of PHAs through irradiation in order to improve their overall properties was also employed in other studies [33, 34]. In the work of Masood et al. [33], vinyltriethoxy silane (VTES) functionalized sepiolite (SP) was incorporated into PHB polymer. PHB was gamma-irradiated in order to investigate the effect of irradiation-induced crosslinking. The preparation of the nanocomposites was carried out by using solution casting, by employing chloroform as a solvent. The onset degradation temperature (T_{onset}) for the nanocomposite (PHB/SP 95/5 w/w) increased from 252°C (PHB) to 271°C, while the gamma-irradiated sample (*viz*. 25 kGy irradiation dose) further increased T_{onset} to 283°C. The latter was ascribed to the development of radiation-induced crosslinked network by improving the barrier effect and delaying the degradation of the nanocomposites. Elsewhere [34], it was reported that the presence of cloisite 30B (C30B) (3wt%) in PHBV/PLA (50/50w/w) blend, increased the degradation temperatures at: 5, 10 and 50% weight losses and maximum degradation (T_{max}) from: 276, 282, 312 and 287/362°C for the PHBV/PLA to: 286, 291, 320 and 299/363°C for the nanocomposite samples, respectively. However, there were no significant changes in the thermal stability of the irradiated nanocomposite samples when compared to neat nanocomposites samples. This is because the C30B absorbing volatile products were emitted during the thermal degradation process and at the same time acted as an insulating barrier towards the e-Beam radiation. In the case of the crystallization behaviour, it was found that the presence of C30B, slightly reduced the: T_c, T_{cc}, ΔH_c, ΔH_{cc}, T_m and ΔHm when compared to the PHBV/PLA blend. This is due to the modification in the macromolecular arrangements of PHBV and PLA chains in the presence of C30B. When the nanocomposites were exposed to e-Beam radiation, the melting temperatures shifted to lower temperatures, while the melting enthalpy significantly increased. On the other hand, the crystallization temperature shifted to lower temperatures and crystallization

enthalpy was slightly increased. This was attributed to the defect caused by the e-Beam irradiation. The increase in the melting enthalpy was related to the phenomenon called, chemi-crystallization, i.e., the increase in crystallinity as a result of the liberation of macromolecular fragments. A follow-up study, based on the same composition, but in the presence of compatibilizer (5wt% PHBV-*g*-MA) and gamma radiation of 100 kGy, was conducted by the same group [35]. The same behaviour in the case of the thermal stability, was recorded with increases in: T_5, T_{10} and T_{50} with approximately 10, 9 and 8°C, respectively due to the presence of C30B, with its inorganic layered silicates restricting the diffusion of volatile degradation products counterbalancing the degradation effect. However, there were no changes after the irradiation process and in the presence of compatibilizer. The same crystallization behaviour was reported, *viz*: T_c, T_{cc}, ΔH_c, ΔH_{cc}, T_m and ΔH_m were slightly reduced in the presence of C30B and γ-irradiation exposure because of the decrease in the arrangement of the PHBV and PLA polymer chains. Two fillers with different treatments were studied by Khandal, Pollet and Avérous [4]. The nanocomposite, based on poly(3-hydroxybutyrate-co-4-hydroxybutyrate) and montmorillonite (MMT) or sepiolite (Sep) nanoclays as well as their treated counterparts (OM-MMT and OM-Sep), were investigated. It was found that the thermal stability increased with increase in nanoclays content. The thermal mechanism for both clays was proposed to be similar. In the case of the MMT, it was reported that the platelets dispersion and formation of a tortuous path retarded the progress of the gases in the material, together with greater char formation at the surface, which in turn, acted as a local insulator. On the other hand, sepiolite increased the thermal stability behaviours from tortuosity of the gases in the material through zeolitic channels. Sepiolite often migrates to the surface where it acts as a barrier for heat transfer, while preventing the volatilization of degradation products and the diffusion of oxygen to the polymer. There was no significant difference in the thermal stability of the treated MMT and the OM-MMT clays, however OM-MMT degradation displayed a degradation taking place at a larger temperature range (higher temperature at the weight loss). This was related to two antagonistic effects, *viz*: (i) the organomodifier catalyses the matrix degradation and thus, decreasing its thermal stability, (ii) the surfactant improves the dispersion and exfoliation of the clay in the matrix, which in turn improves the thermal resistance through a conventional phenomenon of increase in the tortuosity and thermal barrier. These resulted in limited improvement in the thermal resistance. Interestingly, sepiolite-based nanocomposites performed better than the MMT counterparts at the same filler content. In the case of the modified sepiolite, the same two possible mechanisms for the MMT were also proposed. It was reported that clay migrates to the surface of the matrix during heating and act as gas and heat barrier. The thermal properties of cellulose nanowhiskers (CNW) as reinforcement of the PHAs were also reported by other researchers [28, 29]. The positive effect on the thermal stabilization of PLA-PHB bionacomposite fibers, plasticized with ATBC by cellulose nanowhiskers (1wt%), was recently reported by

Arrieta et al. [29]. A comparison between hydrochloric (CNW-H) and sulphuric (CNW-S) acid extracted cellulose nanowhiskers reinforced PHBV was reported [28] elsewhere in the literature. Since these treatments introduce different functional groups on the surface of the CNWs, nanocomposites based on CNW-H were better thermally stable than the CNW-S-based nanocomposites. This is due to the sulphates groups on the surface of the sulphuric acid extracted nanowhiskers (CNW-S), in which their elimination requires less energy, which in turn, promotes and/or catalyzes the degradation of PHBV. The initial decomposition temperature (T_0) and maximum decomposition temperature (T_{max}) increased by 21.4 and 17.6°C, following the addition of 1 wt% CNW-H when compared to the neat PHBV. For the 12 wt% CNW-H reinforced PHBV, the T_0 and T_{max} increased by ~58.1 and ~52.1°C, respectively.

6. APPLICATIONS

6.1. Biomedical Applications

PHAs can be utilized in different applications, such as: food, packaging and household to biomedical because of the wide range of polymers available in the PHAS family, with various properties. The biodegradability and non-toxicity of PHAs open doors for its application, especially in the medical field. In addition, some of PHAs, i.e., P(4HB) have been approved by US Food and Drug Administration for its use in sutures with regard to safety and biodegradability [36]. Table 3 lists all the biomedical applications of PHAs and their blends as well as nanocomposites. PHAs have been exploited in tissue scaffolds to promote growth of the cells by supplying nutrition. These polymers qualify for this purpose due to their unique features, such as biodegradability and good mechanical strength. A wide variety of PHAs have been used as replacement for skin tissue [37], cardiovascular tissue [38], heart valve tissue [39], nerve conduit tissue [40], oesophagus tissue, cartilage and bone tissue [41], as summarized in Table 3. In the case of bone tissue engineering, PHAs are generally of significance, since they have unique properties that are suitable for bone scaffolds, such biodegradability, biocompatibility, vascularization and sufficient mechanical strength [36, 42]. However, the mechanical properties may vary in the PHAs family, hence blending and the addition of fillers have been the subjects of medical research for the past decades. From these studies, it possible to add fillers/polymers, not only to improve the mechanical properties, but to produce multifunctional scaffolds necessary to promote tissue regeneration e.g., as drug delivery carriers. In the same way, for skin tissue engineering, the combination of functionalization of PHAs and preparation methods have been major topics to come up with scaffolds that can mimic the natural healing process. For example, electrospinning processing, offers the advantage to produce fibres with diameters, ranging from 10 nm to

several thousand nanometers. The resulting tissue scaffolds have unique properties, such as: high surface-to-weight ratio, high porosity and tuneable mechanical properties, which afford their applications in wound dressing. For instance, electrospun poly(3-hydroxybutyrate/4-hydroxybutyrate) nanofibers were fabricated by Shishatskaya et al., [37] in order to evaluate its wound properties with Graft-elastic non-woven membrane, carrying fibroblast cells derived from adipose tissue multi-component mesenchymal stem cells.

Table 3. PHAs, Blends and nanocomposites for biomedical applications

Material type	**Application**	**Refs.**
Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) (PHBVHHx)	Bone marrow	[43]
Poly(hydroxybutyrate-*co*-hydroxyhexanoate) (PHBHHx	Bone marrow	[44]
Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) (PHBVHHx)	Bone marrow	[45]
Polyhydroxybutyrate/hydroqyapatite	Bone tissue	[46]
Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate PHBV microsphere/45S5 bioactive glass	Bone tissue	[41]
Poly(hydroxybutyrate)(PHB)/Poly(hydroxybutyrate-*co*-hydroxyhexanoate) (PHBHHx)	Cartilage tissue	[47]
Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) (PHBVHHx)/Poly (propylene carbonate) (PPC)	Blood vessel	[48]
poly(3-hydroxyoctanoate) (PHO)/bacterial cellulose nanofibers	Blood vessel	[49]
Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV)/ poly(L-D,L-lactic acid) (P(L-D, L)LA)/poly(glycerol sebacate) (PGS)	Myocardial patch	[39]

It was pointed out that the membranes were able to facilitate wound healing by protecting it from external influences. From morphological, histological and molecular methods, it was shown that the membranes, combined with cells synthesizing growth factors, sufficiently facilitated wound healing, neo-vascularization and regeneration and enabled wound healing by day 14, following post-surgery. It was demonstrated that it is possible to use nonwoven (electrospun nanofibers) of degradable P(3HB-*co*-4HB) as atraumatic wound dressings that can reduce inflammation, enhance the angiogenic properties of the skin and facilitate its healing.

6.2. Packaging

Over the past two decades, there has been an ever-increasing interest in the reduction of the negative environmental impact caused by food packaging. This has spurred a lot of interest from scientist to come up with a suitable way (or ways) to reduce the petroleum-based plastics with novel biodegradable materials. PHAs, their blends and nano-

composites were exploited as suitable alternatives to replacement of petroleum-based plastics. Table 4 lists selected studies on PHAs and their blends as well as nanocomposites. In order for the product to qualify for packaging, there are several aspects that need to be met, such as: good mechanical properties, good barrier properties and biodegradability. Electrospun blend of PHB and PLA (25:75), plasticized with ATBC displayed good elongation-at-break that is essential for packaging application. The presence of ATBC improved the interaction between PLA and PHB and also the degradation rate under compositing conditions, according to ISO 20200 standard. On the other, the addition of filler offers advantage to control the degradation rate of the biopolymer films, which is of significance, depending on the packaging application [50]. The addition of organophilic montmorillonite (OMMT) in poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), was found to decrease the rate of PHBV degradation [50]. The degradation rate of the PHAs films do depend on various aspects, such as: essential properties of materials (composition, structure, molecular weight), processing of the material (type of processing, surface characteristics), physico-chemical parameters of the ecosystem (temperature, pH, oxygen content and nutrient supply) and microbial parameters of the ecosystem (population density, microbial diversity and microbial activity) [50, 51]. For example, in the blending of PHB with plasticizers, glycerol, tributyrin, triacetin, acetyltriethylcitrate, acetyltributylcitrate and nucleation agent, saccharin were tested under different compositing conditions i.e., soil, river and aerobic [51]. In the case of a soil compost, the samples were found to be covered by: Si, Al, Na, Mg, Cl, K and Ca from soil mineral and soil microorganisms and densely populated areas hypha of fungi, bacteria and others were found. However, microorganisms were able to enter the pores, which is essential for degradation. On the other hand, in river water, there were visible degraded surfaces, similar to soil composting, but the degradation rate was higher than the soil burial test. There were densely populated areas where bacteria formed a layer of organic material (non-identified microorganisms, including algae), covering the surface completely. For aerobic test, by using an aqueous medium and by the determination of oxygen demand in a closed respirometer (ISO9408:1999), it was reported that the degradation after a longer time (60 days) was faster than at a shorter time (40 days). Interestingly, the degradation rate was also higher than those samples for soil burial test. These kinds of tests are important in order to have an idea on how to decompose the packaging films. Of interest, is the inclusion of plasticizers and nucleation agents, which leads to a lower glass temperature and lower crystallinity with numerous, small and imperfect crystallites resulting in the impact strength and the elongation-at-break increasing and the yield stress reducing, which is essential for food packaging. One of the most valuable properties for packaging is the barrier properties of the manufactured films. Still on plasticizers, Jost and Langowski [52] found that plastization of PHBV with triethyl citrate and polyethylene glycol was a more effective method to improve, not only the barrier properties, but also the thermal and mechanical properties.

Table 4. PHAs, Blends and their nanocomposites for packaging applications

Material type	Key features	Refs.
Poly(3-hydroxybutyrate)(PHB)	It showed good barrier to light and easily degradable in different environments	[54]
PLA-PHB (75/25 w/w) blended with D-limonene	Blend with addition of D-limonene renders transparent flexible film with enhanced oxygen barrier and water resistance, biodegradable in compost, hence suitable for food packaging	[55]
Polyhydroxybetyrate (PHB)/poly caprolactone (PCL)/Kaolite ($Al_2Si_2O_5(OH)_4$)	Blending with PCL improved the dispersion of the fillers. The presence of filler enhanced crystallinity and barrier properties to oxygen, D-limonene and water	[56]
PLA–PHB/ATBC	Plasticized blend showed higher tensile strength and far stretchability than PLA with comparable Young modulus and compostable	[57]
PLA-PHB plasticized with polyester plasticizer (Lapol 108)	Elongation at break improved by the presence of plasticizer	[58]
PLA-PHB/carvacrol (antibacterial agent) plasticized with oligomer of the lactic acid (OLA)	The addition of antibacterial agent could improve shelf life and quality of foods by restricting the growth and spoilage by microorganisms	[59]
PLA–PHB/ATBC loaded with catechin (1wt %)	Increased tensile strength and modulus at the expense of elongation at break and the biocomposite disintegrated in composting conditions at laboratory scale level according to the ISO 20200 standard	[60]
PLA-PHB/cellulose nanowhiskers	Good mechanical properties and water resistance, reduced oxygen and UV light transmission as well as appropriate disintegration under composting conditions using ISO 20200 standard	[61]

On the other hand, nanoparticles can be added into polymers to improve the barrier properties of the resulting packaging films. The addition of organo-modified montmorillonite (OMMT-Cloisite 10A) (1wt%) into PHBV and PHB, decreased water vapour permeability by 25 and 41%, respectively [15]. Different permittivity tests on PHBV reinforced nanoclay nanocomposites were conducted by Crétois et al. [53]. Gas permittivity (P) for oxygen (P_{O2}), nitrogen (P_{N2}) and carbon dioxide (P_{CO2}) were carried out on neat PHBV and PHBV/C30B nanocomposites as well as water permittivity. It was pointed out that there were several parameters that influence the barrier properties of the nanocomposites, such as: the extent of dispersion and orientation of nanoclays, nature of permeated molecules, the quality of the matrix/nanoclays interface and the degradation effects induced during the melting process. In general, these studies demonstrated that PHAs can be exploited for packaging by overcoming some of their disadvantages by blending with other biopolymers and/or nanofillers.

CONCLUSION

Over the years, the approach of synthesizing different blends and composites of PHAs have been greatly investigated. This is as a result of the need to overcome the drawbacks associated with the use of these polymers individually. The major reason behind adopting the methodology of combining two or more PHAs is to effectively improve the overall properties and characteristics. This approach has led to the synthesis of PHAs with greater efficiency when compared to individual PHAs. In addition, it has made it possible for PHAs with specific characteristic that exactly suits a particular application to be developed. Furthermore, this approach has provided greater opportunity for the application of PHAs in different fields because the individual properties of the blends and composites are tapped into, maximally. Although, the cost still remains a major down side associated with the synthesis of blends and composites of PHAs. However, thus far, various blends and composites of PHAs have been developed and studied. Successful application of these blends and composites of PHAs in fields ranging from industrial to medical application, have been reported. However, there are still further investigations on new blends and composite of PHAs for potential applications in various fields.

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Chapter 8

POLYHYDROXYALKANOATES: BIODEGRADABILITY, RECENT AND POTENTIAL APPLICATIONS IN PACKAGING

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are becoming very popular in the biodegradable polymer market. This is because of their promising properties, such as high level biodegradability in different environments. Amongst the well-known biopolymers, these biogenic polyesters (Image 1) (PHAs) emerge as potential suitable and sustainable replacements for fossil fuel-based thermoplastics. PHA can be produced from bacteria

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cell and then formulated and processed by extrusion for the production of rigid and flexible plastics. The applications of PHA, include: packaging, moulded goods, paper coatings, non-woven fabrics, adhesives, films and performance additives. The present chapter reviews the different classes and applications of PHAs, which include: industrial, agricultural, with specific focus on potential applications of PHAs in packaging.

Keywords: polyhydroxyalkanoate, biopolymers, polyesters, packaging, fossil fuel

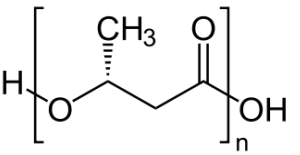


Image 1. Chemical structure of Polyhydroxyalkanoates.

1. INTRODUCTION

Recently, concerns have arisen all over the world, over the problems associated with the accumulation in the environment, of non-degradable plastics and the management of solid wastes, generally. This has led to the great interest in the development of plastics that are biodegradable and at the same time possess the physical, mechanical and chemical properties of conventional plastics. One of such plastic materials that has been developed is polyhydroxyalkanoates (PHA) [1-3]. The occurrence of PHA in the cell of bacteria was first reported by Beirjerinck in the year 1888 [4]. As at that time, PHA was studied by many biochemists and they referred to them as lipid. However, it was later proven from research that this "unknown" material found in the cell of *Bacillus megaterium* was a homopolymer of hydroxyacid. They are thermoplastic polyesters of hydroxyalkanoates (HAs) produced by a variety of bacteria and fungi as intracellular carbon and energy storage compounds, under unbalanced growth conditions. They are mostly produced by microorganism (*gram-negative* and *gram-positive* bacteria) in an environment that has a lot of carbon and they (PHA) are deposited in the cytoplasm of cells as granules [5, 6]. PHA are accumulated in cells to levels as high as 90 percent of the cell's dry weight [7]. Over 75 different generations of bacteria have been found capable of synthesizing PHAs. Bacteria, including: *Alcaligenes eutrophus, Alcaligenes latus, Azotobacter vinelandii,* methylotrophs, pseudomonads and recombinant *Escherichia coli*, have been used for the production of PHAs and a greater than 2 g PHA/Uh productivity has been attained [5]. There are currently about 150 hydroxyalkanoic acids of different types that have been discovered to be constituents of the bacterial storage polyesters [8]. The first member of the PHAs family that was first discovered in *Bacillus megaterium* by Lemoigne in 1926, is PHB [9-11]. It is the best characterized and the most widely studied member of the PHA family. Since the discovery of PHB, other members of the PHA family have been discovered and their

advantages and benefits over conventional polymers, have been greatly explored as well as their limitations [6]. Through an advance understanding of molecular biology, genetics and metabolic processes of PHA synthesizing bacteria, as well as gene cloning, different recombinant strains have successfully synthesized polyesters with different monomer units and or were able to amass more polymer [5]. In addition, plants that are genetically engineered to harbour the gene of a bacteria that can synthesize PHA commercially, are being developed. Furthermore, the ability to modify the functional groups (such as: hydroxyl, carboxyl, epoxy and halogens) attached to PHAs creates an avenue to specifically adjust their thermal and mechanical properties [12]. The molecular mass of PHA is between the range of 50,000-1,000,000 Da and this is dependent on the PHA producer. From studies, PHAs are known to belong to the family of biodegradable polymers and they are bio-based [6]. These properties distinguish PHAs from petroleum-based plastics because their exposure to soil as well as compost or marine sediment leads to degradation. The degradation of this group of biopolymer is dependent and affected by factors, such as: the microbial activity of the environment, the surface they are exposed to, moisture, temperature, pH, molecular weight, polymer composition, crystallinity and the nature of the attached monomer unit of the PHA [5, 13]. For example, a more rapid degradation is observed in copolymer of PHAs containing PHB when compared to 3HB-*co*-3HV copolymers. PHAs are also known to be very environmentally-friendly when compared to polymers that are chemically synthesized. Hence, they serve as a solution to the environmental and social problems created by plastic industries, associated with the use of conventional polymers [12]. Although PHA offers good properties that make them advantageous for use as biodegradable plastics, certain characteristics still hinders high volume applications of PHAs. Some of these draw backs, include: pronounced brittleness, very low deformability, high susceptibility to rapid thermal degradation, difficulty in processing them like the conventional thermoplastic, as a result of their quick thermal degradation behaviour and their high cost of production [14]. However, with the development of bacteria strains or plants that are capable of efficiently synthesising PHA, the cost of production is expected to reduce thus making PHA more competitive with conventional polymers [5]. Also, by copolymerizing PHB (Polyhydroxybutyrate) with valerate, an improved toughness and an acceptable loss of strength/modulus has been achieved [14]. Hence, some of these limitations have been successfully overcome thus, polyhydroxyalkanoates have become better substitute for polymers derived from petroleum [15]. Currently, PHAs are employed in a number of applications in: medicine, agriculture and packaging amongst others. These group of biopolymers are used as carriers and matrices for controlled release of nutrients, fertilizer in the agricultural sector as well as for the controlled release of active pharmaceutical compounds or ingredients. In addition, it is used for the manufacture of surgical implants, sutures, pines, etc. Furthermore, they are used for producing packaging materials. PHAs are considered to be promising “reserve materials” that is worth been investigated, to a very large extent. The

production and preparation of PHAs on a large scale is also been carefully looked into in several regions of the world.

2. CLASSIFICATION OF PHAS

PHA can be generally classified, based on their structure, i.e.: the numbers of the carbon atoms present in the monomeric units viz; short-chain-length (SCL) PHAs and medium-chain-length (MCL) PHAs, which consist of between 3–5 and between 6–14 carbon atoms, respectively [16]. PHAs can further be grouped as: homopolymers (consist of either scl-PHAs or mcl-PHAs) or copolymers (consist of a blend of different monomers of scl-PHAs and/or mcl-PHAs) [12, 17-19]. Shown below in Table 1, are different types of PHAs and their classifications.

Table 1. Showing examples of PHAs and their classification

Types/Examples	Classification
Poly(3-hydroxybutyrate) P(3HB)	Short-chain lengths PHAs (PHA-SCL)/homopolymer
Poly(3-hydroxyhexanoate) P(3HHx)	Medium-chain lengths PHAs (PHA-MCL)/homopolymer
Poly(4-hydroxybutyrate) P(4HB)	Short-chain lengths PHAs (PHA-SCL)/homopolymer
Poly(3-hydroxyoctanoate) P(3HO)	Medium-chain lengths PHAs (PHA-MCL)/homopolymer
Poly(3-hydroxybutyrate-*co*-3 hydroxyhexanoate)	Hybrid copolymer of Short-chain lengths PHAs (PHA-SCL) and Medium-chain lengths PHAs (PHA-MCL)
Poly(3-hydroxyvalerate) P(3HV)	Short-chain lengths PHAs (PHA-SCL)/homopolymer
Poly(3HH$_x$-*co*-3HO)	Medium-chain lengths PHAs (PHA-MCL)/copolymer
Poly(3HB-*co*-3HV)	Short-chain lengths PHAs (PHA-SCL)/copolymer

Both classes of PHAs are produced by PHA synthases, but the difference between the two classes is the substrate specificity of the PHA synthases enzyme. The enzyme is able to accept 3HAs of a particular range of carbon length [20]. For instance, PHA synthase of *Alcaligenes eutrophus* is able to polymerise 3HAs, consisting of 3–5 carbon atoms while that of *Pseudomonas oleovorans* is able to accept only 3HAs of 6–14 carbon atoms [20]. Recently, it has been discovered that monomers with various functional groups, such as: halogen, hydroxyl, epoxy, cyano, carboxyl and esterified carboxyl groups on the chain are in mcl-PHAs [21]. Another group of PHA that has been produced from alkanoic acids of odd carbon numbers, by recombinant strain is the terpolymer. An example of this, is poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxy-heptanoate), synthesized from the recombinant strain of *A. eutrophus*, harbouring the PHA synthase gene of *Aeromonas caviae* [22, 23].

2.1. Structure of PHAs

The general formula [24] of the monomer unit of PHA is [O-CH(R)-CH_2-CO]. Due to the stereo-specificity of the polymerizing enzyme (PHAs synthase), the (R)-3HA monomer units are all found in the rectus configuration (R configuration) [24]. Polyhydroxyalkanoates possess branched, straight, saturated, unsaturated and aromatic structures [25, 26]. Various structures exist for the different types of PHA that have been discovered thus far. However, Figure 1 is the general and generic structural formula for all types of PHAs.

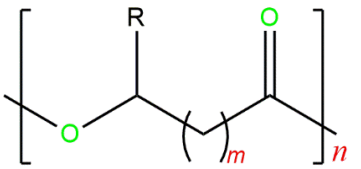
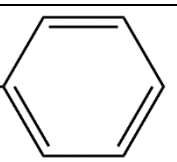


Figure 1. General structure of PHAs.

The value of the integer *m*, is typically 1; however, exceptions include: 4-hydroxyalkanoates such as 4-hydroxybutyrate and the degree of polymerization is quantified by integer *n*. The functional group (R group) varies from one type of PHA to another [27]. The table below shows the functional groups attached to some different PHAs.

Table 2. Showing some of the functional groups attached to PHAs

Functional group (R group)	**Type of PHA**
—CH_3	Poly(3-hydroxylakanoate)
—CH_2—CH_3	Poly(3-hydroxyvalerate)
—$(CH_2)_2$—CH_3	Poly(3-hydroxyhexanoate)
—$(CH_2)_4$—CH_3	Poly(3-hydroxyoctanoate)
—$(CH_2)_6$—CH_3	Poly(3-hydroxydecanoate)
—CH_2— 	Poly(3-hydroxy-5-phenylvalerate)

The structures below (Figures 2 and 3) are different examples of short chain length PHAs and medium chain length PHAs, respectively.

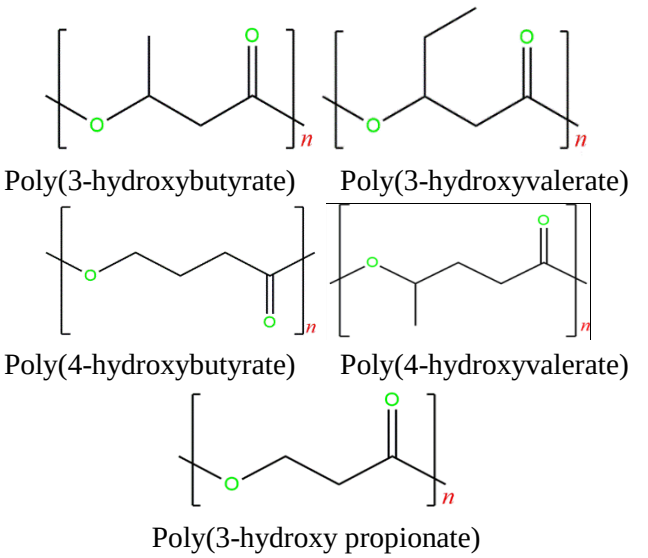


Figure 2. Structures of some short chain length PHAs.

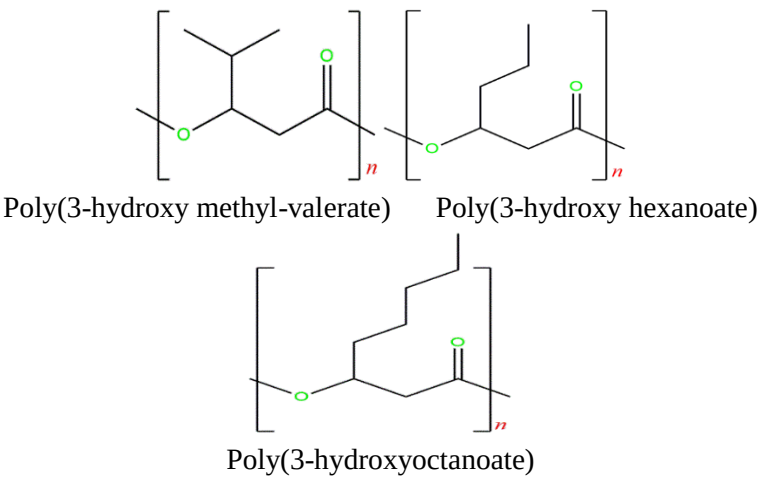


Figure 3. Structures of some medium chain length PHAs.

2.2. Properties of PHAs

Generally, many different types of PHAs that exist, exhibit similar properties to petroleum-based polymers such as polypropylene, especially thermal and mechanical properties [28-30]. The length of the monomer side chain of any member of the PHA family, influences the thermal and mechanical properties [30]. For example, homopolymer that consists of short side chain length monomers, e.g., PHB, exhibits

stiffness and brittleness, whereas a copolymer that consists of monomers of different chain lengths, such as poly (hydroxybutyrate-*co*-hydroxyhexanoate) (P(HB-*co*-HHx), is pliable and flexible [30]. These properties have caused them to gain a lot of attention. The physical characteristics of PHAs may vary from one another and this is basically dependent on the composition of the monomeric unit that makes up the PHA [31]. However, the following are the general physical characteristics of PHAs [32]:

- Hydrophobic, i.e., it is not soluble in water
- Biodegradability and biocompatibility
- Soluble in chloroform and chlorinated hydrocarbons
- Non-toxic in nature
- Good resistant to ultra-violet rays
- Poor resistance to acids and bases
- Less sticky when melted in compared to traditional polymers
- Ability to sink in water hence anaerobic biodegradation is facilitated
- Stiff, brittle and semi rubber-like
- Good water vapour barrier properties
- High degree of polymerization
- Optically activity
- Isotactic, that is they have stereo-chemical regularity in repeating units
- Good fat and odour barrier properties useful in food packaging.

2.2.1. Thermal and Mechanical Properties

Generally, the family of PHAs possess thermal and mechanical properties that vary considerably from one another [15]. These differences in the thermal and mechanical properties depends on the component of the monomer that makes up the PHA. Short chain length PHAs are brittle, stiff and have a high degree of crystallinity of between 60-80% while medium chain length PHAs are elastic in nature, flexible, have low melting temperature, low degree of crystallinity and low tensile strength [4, 30]. The basic properties of members of the PHA family are explained by considering the properties of PHB because it is the most widely investigated member of the PHA family.

2.2.1.1. Properties of PHB

P(3HB) is a water insoluble biopolymer with relative resistance to hydrolytic degradation, its stereo-regularity makes it highly crystalline in nature. It possesses low oxygen permeability and very good thermoplastic properties. When compared to the petroleum-based polymers, e.g., polypropylene, its mechanical properties, such as Young's modulus and tensile strength are poor [30]. It is very brittle and stiff, the amorphous and crystalline forms of PHB, have densities ~1.26 and ~1.18 g/cm^3. The factors responsible for the brittleness of PHB, include: closeness of its' glass transition

temperature to that of room temperature and low nucleation density. Therefore, large spherulites exhibit inter-spherulitic cracks. In addition, the secondary crystallization of the amorphous phase takes place during storage at room temperature [32]. The range of the molecular weight of P(3HB) produced from wild type bacteria, is usually between the range of 10,000-3,000,000 Da. P(3HB) is optically pure, has a poly dispersity index of around two and possesses piezo electricity. Whereas, P(4HB) is a strong and pliable thermoplastic material, its elongation-at-break is ~100%, thus, it is very elastic in nature and it has a tensile strength that is close to polypropylene [33]. When other HA monomers, such as 3-hydroxyhexanoate, 3-hydroxypropionate and 4-hydroxybutyrate are incorporated onto the polymeric chain of P(3HB), copolymers are formed and this significantly improves the material properties of P(3HB). The material properties of P(3HB), such as: crystallinity, melting point, stiffness and toughness are greatly enhanced. An example of this type of copolymerization strategy is the most common copolymer, P(3HB-*co*-3HV). It is more flexible (due to a decrease in the Young's modulus), has lower melting point temperature, lower crystallinity, decreased stiffness and increased elongation-at-break when compared to P(3HB). The thermomechanical properties of the copolymer are determined by the percentage mole of the 3HV and this mole percentage can vary from between 0-30mol% 3HV [21]. Furthermore, it has been discovered that PHA terpolymers are better materials when compared to copolymers. That is, the addition of more than a secondary monomer results in better and improved thermal and mechanical properties of PHAs. An example is P(3HB-co-3HV-co-3HHx) terpolymer that is more amorphous in nature, has no melting peak and a very increased elongation at break (408%) [34, 35].

According to Bugnicourt et al, the general range of the thermal and mechanical properties of PHAs are tabulated below [32, 36].

Table 3. Showing the range of the properties of PHAs

Property [units]	**Values**
Melting temperature T_m [°C]	160–175
Young's modulus E [GPa]	1–2
Oxygen transmission rate OTR [cc·mm/m^2/24 h]	55.12
Glass transition temperature T_g [°C]	2
Crystallinity degree X_{cr} [%]	40–60
Water vapour transmission rate WVTR [g·mm/m^2/24 h]	2.36
Elongation at break ε [%]	1–15
Tensile strength σ [MPa]	15–40
Crystallinity [%]	80
Molecular weight [Daltons]	5 x10^5
Density [g/cm^3]	1.07-1.25

3. Requirements for PHAs in Food Packaging

3.1. General

It is well known that ~125 million tons of plastics are used throughout the world for packaging purposes [37]. It is clear that there is a huge potential market for bio-based materials for packaging applications if they are produced with appropriate processability, functionality and at a reasonable price. This has been the biggest challenge for producers of bio-based materials for packaging. The main reason for this is that bio-based materials have to compete with the synthetic plastics, which are cheaper, easy to process and with significant optimized packaging properties. Bio-based materials for food-packaging are materials derived from renewable sources. These types of materials can basically be used food packaging. Bio-based plastics have been divided into three main classes, based on their origin and production (Figure 4). Category 1 PHAs are polymers that are directly extracted from biomass. Polymers produced by chemical synthesis by using renewable bio-based monomers belong to category 2, whereas polymers produced by micro-organisms or genetically modified, fall under category 3.

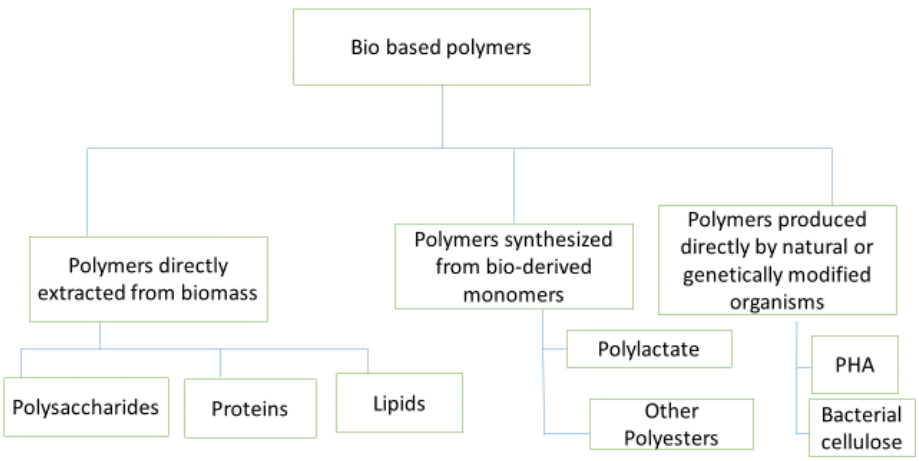


Figure 4. Different types of biologically based materials [37].

Poly(hydroxyalkanoates) (PHAs) belong to category 3 and are a family consisting of renewable, biologically degradable, biocompatible and optically active polyesters. Their production occurs through many bacterial species in the form of intracellular particles, operating as an energy and carbon reserve material. The final properties of PHAs depend on the type of substrate and bacteria strains used for the synthesis of the bio polyesters. Figure 1 shows the general structure of PHAs. The presence of functional groups in the side chains of the polymer makes it possible for the polymer to be modified chemically as a result ability of the polymer to be used for food-packaging applications. There are

several factors required for a selected material to fulfill for food packaging, *viz*: (i) enclosing the food, (ii) protecting it from the environment, (iii) maintaining the sensory food quality, (iv) stability under extreme storage conditions, (v) maintaining a modified atmosphere and (vi) food grade quality of the packaging, needed with regards to purity.

In order to ensure that the above requirements are functional, it is important to control the mechanical and barrier properties of the PHAs packaging materials during the experimental storage conditions. In the case of PHAs, as explained earlier, these properties are highly dependent on the structure and monomeric composition of the copolymer [38-41]. In most cases, PHAs are modified, usually, either chemically or enzymatic modification of the polymeric packaging material with the production of blends and composites with other compatible materials. Generally, there are several key factors that make PHA a polymer of choice for food packaging. These factors include: (i) PHAs can easily be processed into an excellent packaging films through thermoforming. This process can be done by using PHAs as a single material or in combination with other synthetic or bio based polymers as a result forming a food package material [42], (ii) PHAs have reasonable degree of crystallinity and elasticity and as a result, they can be molded into different parts, such as flexible foils for wrapping different materials. Based on the adaptable degree of crystallinity, PHAs can be used to form rigid and robust components for storage boxes and containers [43, 44], (iii) due to the hydrophobic nature of PHAs, PHAs films generally show high water vapor barrier [41, 45], (iv) depending on the type of PHAs used, PHAs (*viz* polyhydroxybutyrate and Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) display a high oxygen barrier. This is required to impede the growth of aerobic microbes and the oxidative decomposition of unsaturated fatty acids [46]. Its' well known barrier properties for oxygen, water and carbon dioxide, widen the applications of PHA as a fundamental material for production of bottles for liquid foods as well as any carbon dioxide-containing liquids [43] (v) The high UV-barrier is advantageous in a sense that it protects unsaturated lipid parts in food from formation of radicals as result accelerating their decomposition.

3.2. Comparison of PHA with Other Petroleum Polymers for Packaging Applications

A fair amount of studies [41, 47, 48] have been done to compare the food packaging performance of PHA with other synthetic polymers, such as: PP, PVC, PET, etc. Table 4 summarizes the different properties of biodegradable and commodity polymers. In these studies, PHA prepared with different methods was put into variety of tests, such as barrier and mechanical properties. The performance of PHA with regards to the tests was compared with well-known synthetic polymers for food packaging applications. An experiment was conducted in a study [41] to investigate water, carbon dioxide and

organic solvent transport of PHB and PHBV with HV molar ratios. The authors observed a similarity trend in the performance of the two types of polyhydroxyalkanoates with regards to: CO_2, water and solvent experiments, when compared to well-known plastics, such as PVC as well as PET. In another study [47], the dimensional and mechanical tests of PHB were done and compared with PP under the same experimental conditions. It was found that PHB had a lower resistance to dynamic compression in comparison to PP. The authors from the same study further reported that the sensory test done with selected foods showed no difference to the one of PP. The evaluation of the odor intensity showed values of 5.33 (average strong) and 3.75 (average) for PHB and PP respectively.

Table 4. Comparison of typical biodegradable polymer properties with other polymers [49]

Polymer type	**T_g (°C)**	**T_m(°C)**	**Tensile strength (MPa)**	**Tensile modulus (MPa)**	**Elongation-at-break (%)**
LDPE	-100	98-115	8-20	300-500	100-1000
PCL	-60	59-64	4-28	390-470	700-100
Starch	-	110-115	35-80	600-850	580-820
PBAT	-30	110-115	34-40	-	500-800
PTMAT	-30	108-110	22	100	700
PS	70 to 115	100	34-50	2300-3300	1.2-2.5
Cellulose	-	-	55-120	3000-5000	18-55
PLA	40 to 70	130-180	48-53	3500	30-240
PHB	0	140-180	25-40	3500	5-8
PHA	-30 to 10	70-170	18-24	700-1800	3-25
PHB-PHV	0 to 30	100-190	25-30	600-1000	7-15
PVA	58 to 85	180-230	28-46	380-530	-
Cellulose acetate	-	115	10	460	13-15
PET	73 to 80	245-265	48-72	200-4100	30-300
PGA	35 to 40	225-230	890	7000-8400	30
PEA	-20	125-190	25	180-220	400

The strong odor distinguished by the tasters for PHB samples confirmed that the packaging even after 3 months of injection still retained a certain level of odor. The results generally showed that it is feasible to design packages from bio-based materials such as PHB. Furthermore, an interesting investigation was made with regard to the bio-degradation behavior of the PHAs in comparison to commodity polymers [47]. To be specific, PHB packaging experienced a swift degradation in different environments which is an attractive quality since consumption of processed food requires packaging for conservation is fairly high resulting in an excess of solid residues. However, PP

packaging was reported not to degrade easy, causing accumulation of material in the environment. The superior properties of PHB in comparison to PP with regard to food packaging were further shown by investigating light transmission behavior of the two polymers [48]. It was reported by the authors that PHB had 0% transmittance in the range of 250 to 350 nm, whereas within the same range PP showed minimum value of 20.43 (250 nm) and further showed a maximum of 84.21 (750 nm). The results demonstrated that PHB in comparison with PP can be recommended for use in food packaging which requires ultraviolet light barrier in the range of 220-400 nm, which include packaging of soy oil and milk.

3.3. Barrier Properties

It is common knowledge that the oxygen barrier capability of any food packaging material vessels for fresh food, plays an important role for its conservation. It is clear that in order to maintain the quality of PHA as a packaging material and for it to act as a barrier, it must overcome the driving force of the difference in oxygen partial pressures inside the package (*viz.* 0-2%) against the outside pressure (21%) [50]. According to the oxygen permeability test, a polymer film to be used for packaging application should have a low oxygen permeability coefficient. This means that the oxygen pressure inside the container decreases up to a level whereby the oxidation is restrained and hence increase the shelf-life of the product. Several authors have reported on the oxygen permeability values for different types of PHAs [41, 43, 44, 51]. In most cases, nanofillers were added to different types of PHAs used for food packages in order to improve their barrier properties to gases, vapours and organic compounds. In these studies, it was observed that the interactions between the nanofiller and PHAs, the crystallinity and content of the nanofiller had an effect on improving and/or decreasing the oxygen barrier properties. Pascual and co-workers investigated ZnO-reinforced poly (3-hydroxybutyrate-co-3-hydroxyvalerate) bionanocomposites with antimicrobial function for food packaging. The authors reported a noticeable decrease (Figure 5) in the oxygen permeability of PHBV as the zinc oxide (ZnO) nanoparticle content rises up to 4.0 wt%, demonstrating the impeding capacity of the highly crystalline ZnO. The improved barrier performance was attributed to the homogenous dispersion of ZnO and its strong interfacial adhesion with the polyester matrix that causes chain immobilization, as a result of a decrease in the overall diffusion. However, increasing the nanoparticle content up 8.0 wt% did not improve (Figure 3) the oxygen barrier performance, despite a better interaction between the ZnO and polyester matrix. According to the authors, this is due to the nanoparticle clustering at this content, which has led to the formation of preferential paths for the permeants to diffuse faster. Similar behaviour was reported for

PHBV nanocomposites-filled with clay [52], where the authors reported a significant decrease of up to 5.0 wt% nanofiller loading and then remained unchanged.

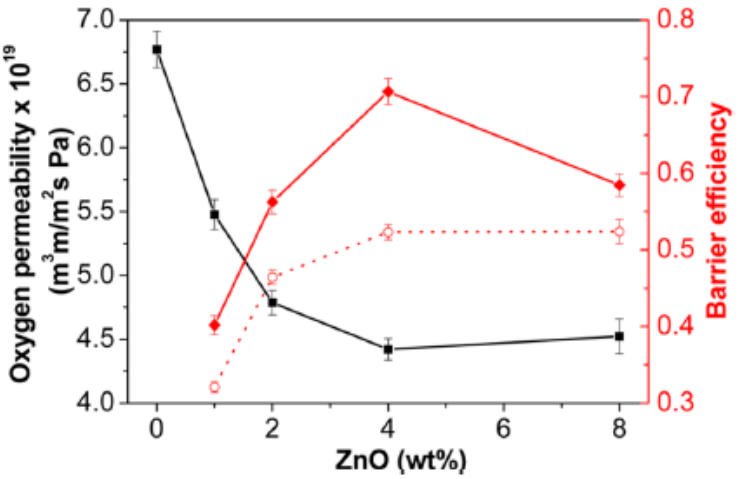


Figure 5. Oxygen permeability and barrier efficiency for water (solid diamonds) and oxygen (open circles) [51].

One of the advantages of PHA polyesters from a chemical point of view is their distinguished hydrophobicity when compared to other biopolymers, such as starch. The water vapour barrier is determined by the water vapour permeability coefficient (WVPC) or water vapour transfer rate (WVTR) which denotes the amount of water vapour that spreads per unit area and time, in or out of a packaged material. Generally, water barrier depends on the type of food that is being packaged. This means that there are those types of food that require low and/or high water barrier. For example, for fresh food products, it important that moisture is not dried out, whereas for the bakery products which are easily attracted by fungal infections in molds, it is important to avoid water flow. A couple of fillers, *viz:* MWCNT, nanoclay, cellulose, carbon fiber, graphene and ZnO, were added into different PHAs matrices in order to improve the water barrier properties [51, 53, 54]. It was reported, from this studies that the addition of nanofillers improved the water barrier properties of the nanocomposites in comparison to neat biopolymer. It was further observed that the improvements in water barrier properties were larger in ZnO/PHBV nanocomposites than those reported for MWCNT or carbon fiber reinforced PHBV and was comparable to those of nanoclay composites. The performance of ZnO/PHBV nanocomposites emerged from the competition of water affinity, degree of crystallinity and tortuosity effects. It appears from the study [51] that the hydrophilic nature of the nanoparticles is normally overweighed by the increase in crystallinity and level of tortuosity as a result the overall decrease in the water uptake and water vapour permeability (WVP). It was reported in the same study [51] that the nanocomposites with the highest loading of ZnO nanoparticles showed reductions in both parameters (water

uptake and water vapor permeability) was smaller than that of the sample with 4 wt% (Figure 6) due to the lower crystallinity and that the presence of small nanoparticle clusters, which resulted in preferential penetrant pathways that have undesirable effects on the barrier performance. It was also reported in the literature that the morphology, functionalization and the content of the filler also played a critical role in determining the water vapour barrier properties [55]. Farahani et al. investigated the water vapour barrier properties of modified Cloisite 30B/poly (3-hydroxybutyrate-co-3-hydroxyvalerate) composites. There was a significant reduction in the water vapour transmission rate (WVTR) associated with modified composites (PHBV/PHB-C30B) in comparison to the unmodified PHBV/C30B. The decrease in WVTR associated with (PHBV/PHB-C30B) nanocomposite samples was attributed to a lower degree of agglomeration and possible exfoliated morphology, especially at less than 10 wt% clay content. Nanocomposites with higher clay content showed a combination of intercalated and agglomerated structures, which caused an increase in the effective path length for the diffusion of water vapor through the polymer matrix. The water vapour resistance decreased for the unmodified clay nanocomposites with increase in the clay content. This may be due to the agglomerated clay nanoparticle in the PHBV matrix. Generally, the agglomeration of clay mineral facilitates the entry of water molecules inside the polymer nanocomposite films. The high water vapour resistance characteristics of the modified composites can be considered as a highly effective potential green-based packaging alternative to the current synthetic polymer-based packaging materials.

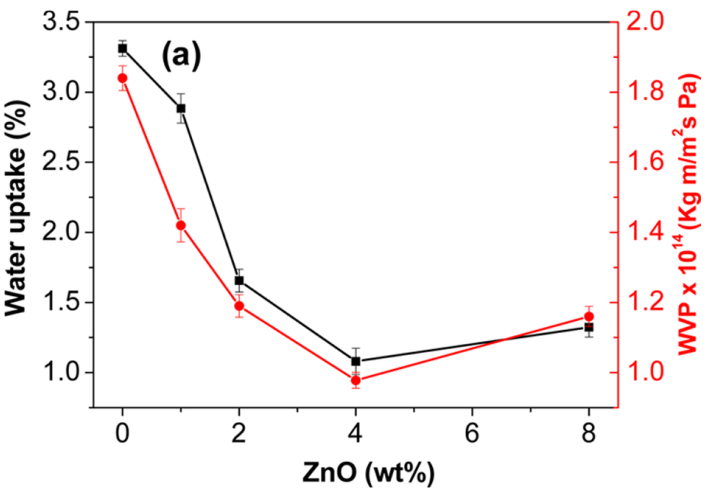


Figure 6. (a) water uptake and water vapor permeability (WVP) PHBV/ZnO nanocomposites as a function of ZnO content [51].

It was reported in the literature [56] that the type of filler used, can have a significant influence on the barrier properties of the resultant PHAs. The authors reported an increase in water vapour transmission rate (Figure 7) of the samples, proportionally with

the addition of cellulose fibers (CF) in the PHB matrix. The results confirm that the addition of CF is not effective in order to improve the barrier properties of PHB.

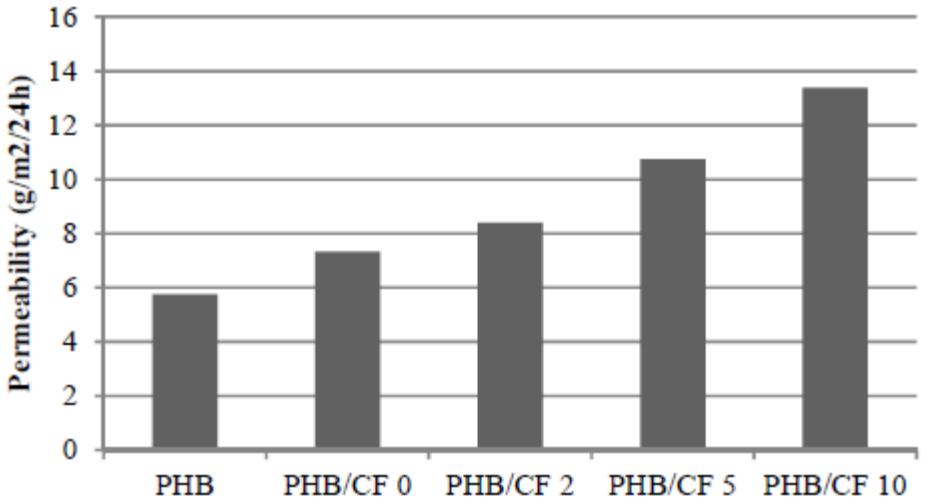


Figure 7. The effect of cellulose fiber over the permeability of the tested PHB composites and neat PHB [56].

Table 5. Barrier properties of PHAs/nanoparticle system

PHAs/nanoparticle systems	**Methods of preparation**	**Summary of the barrier properties**	**Ref.**
Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate)/cellulose	Solvent casting technique	4 wt.% of cellulose nanocrystals exhibited better barrier properties up to 4 times that of neat PHBV	[57]
Keratin–Polyhydroxyalkanoate	Melt compounding method	1 wt.% of keratin additive reduced water and oxygen permeability of pure polymer to less than half of its value	[58]
Poly(3-hydroxybutyrate)/ZnO	Solution casting technique	The nanocomposites showed reduced water uptake and superior gas and vapour barrier properties compared to neat PHB	[59]
Polyhydroxybutyrate-*co*-valerate (PHBV)/clay	Solvent casting technique	5 wt% of clay enhanced gas and vapor barrier properties and UV blocking performance	[52]
Poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate/sepiolite nanocomposites	Solution casting method	better thermal and water barrier properties for the food packaging industry	[60]

3.4. PHA Packaging in Different Types of Food

3.4.1. Cheese

Cheese is a food derived from milk and it is produced in a range of flavors, textures and forms by coagulation of the milk protein casein. It is respiring and releases carbon dioxide during storage that makes the use of bio-based packaging relevant for cheese packaging. As a result of the respiration of cheese, packaging materials must have a high carbon dioxide transmittance rate, which makes PHA suitable for packaging of this type of food.

3.4.2. Frozen Food or Products

It was explained that PHA materials have relatively low gas and water vapour barriers, which automatically make PHAs polymers of choice for a long term food application, such as the dairy products, i.e., cardboard cartons for milk.

3.4.3. Fruits and Vegetables

The high transmittance of carbon dioxide for PHAs as explained earlier, when compared with oxygen, is interesting in relation to packaging of high-respiring fruits and vegetables.

3.4.4. Non-Carbonated Beverages

PHA, together with PLA makes an excellent bottle, cup, containers, etc. for non-carbonated beverages. It was mentioned that PHA can also be used in coatings for cardboard cartons for milk and juices as a replacement for synthetic coatings. The PHA containers was recently tested as container in juices and the packaging results were positive [37].

3.5. Biodegradation

Another valuable aspect of biomaterials, used in packaging, is their ability to decompose into environmentally compatible fragments. It worth mentioning that the biodegradation of the polymers is affected by several parameters, such as: microorganism present, surface area of the sample, temperature, pH and humidity of the medium and also the availability of oxygen and nutrients in the medium [61, 62]. For instance, it was reported that there was no biodegradation of PHB in portable water, however in the media of organic material, such as residues and sewage, the sample degraded within 90 days [48]. It was also stated that the microbial degradation was gradual and uniform for samples buried in manure of an aviary bed, while there was no change for fat tank (low activity water with low pH). Nevertheless, PHB biodegradation took place within

acceptable and/or recommended time frame for packaging, i.e., 90 days. Blending biopolymers can reduce the biodegradation rate of specimens [63, 64]. This is because one of the polymers may act as nucleating agent hence increasing the crystallinity of the blend. Therefore, the more crystalline the polymer is, the slower is its biodegradation, since biodegradation starts from the amorphous region. In order to balance and/or have control over biodegradation rate, one can add plasticizer to reduce the crystallinity of the polymer and/or blend [63]. It is also recognized that biodegradation rate of PHAs and their blends can be reduced by adding additional filler into the system [65, 66]. Such results are attributed to the hydrophobicity of the filler and its interaction with the matrices. The more the interaction, leads to the filler protecting the matrix from the microorganisms and/or enhance trans-crystallization of the polymer matrix, which in turn increases its crystallinity and therefore, the higher the crystallinity, the longer the biodegradation process.

3.6. Migration

One of the most important issues when it comes to PHAs for food packaging application, is migration [48, 67, 68]. This phenomenon depends on the selection of monomers and additives in order to avoid the risk of food contamination. In Bucci et al. [48] report, migration test on PHB packaging components was performed on aqueous food, acid food, aqueous food containing oil/fat, acid food containing oil/fast, oily or fatty foods, alcoholic food and dry solid food. For all these simulants, the results were below the limit recommended by the Resolution 105/99, which is 8.0 mg/dm^2 or 50 mg/kg. Furthermore, the results indicated that the containers produced can be used for products under different storage conditions, such as for refrigerated products and for long-term storage at room temperature. The migration test, based on functionalized cellulose nanocrystals (methy ester (CNC-me) bionanocomposite, was evaluated for migration in isooctane and 10%v/v ethanol [69]. It was stated that the overall migration decreased in the systems with an increase in the amount of CNC-me. Similar study, based on functionalized carbon nanotube as reinforcement in PHBV, on the overall migration in two liquids simulants (isooctane and 10%v/v ethanol) according to EU No 10/2011 standard, was conducted by Yu et al. [53]. The maximum migration level in isooctane and 10%v/v ethanol were 3.0 and 4.2 mg/kg, respectively of the simulants for the nanocomposites containing 7wt% functionalized carbon nanotubes. In summary, the presence of the filler generally, reduces the overall migration; however the main concern is the migration of these nanoparticles into the products. The impact of the nanoparticles due to their nanosize on our health and/or environment is still not clearly understood, which can be a dangerous choice, when using reinforcement in biopolymers for food packaging [70]. Nonetheless, cellulose-based composites performed better than those

based on carbon nanotubes-based composites, which can be a relief since cellulose nano-whiskers are extracted from natural resources [69].

CONCLUSION

Generally, PHAs have shown to be promising biopolymers for a variety of applications. Despite the fact that they can be brittle, several successes in their application have been reported. This is because they have been subjected to different forms of modifications that have led to better and improved properties. These improvements have permitted the development of more flexible grades of PHAs. In addition, other methods of processing PHAs have been devised. These enhanced properties have given them the ability to penetrate the food packaging industry and market for the storage of food products, amongst others. Furthermore, because of the good barrier properties of PHAs, they are used as biodegradable plastics by the packaging industry. This has contributed and helped in solving problems associated with environmental pollution. Hence, it is anticipated that PHAs will be far better candidates as substitutes for conventional thermoplastics in applications, such as packaging. Nonetheless, certain limitations, such as: high cost of commercial PHA, reduced market availability still affect its wholesome takeover. For greater economic viability and sustainability of PHA to be achieved, various researches are still on going. Further research is needed in order to develop new methodologies that will reduce the cost of production, increase and improve the yield of PHA as well as increase the industrial sustainability, thus, allowing for PHAs to be introduced to the broader market.

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Chapter 9

POLYHYDROXYALKANOATES: AN IDEAL POLYMERIC MATERIAL IN FOOD PACKAGING

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ABSTRACT

The ideal polymeric material for direct food contact applications should possess specific characteristics and properties. These properties include the preservative role of the packaging materials against external mechanical damages and bacterial spoilage of the contained food. They must possess the ability to act as gas and liquid barriers providing protection against oxidation and moisture. These types of materials should also be non-toxic, versatile, and compatible with the majority of the food, biodegradable, sustainable, practicable, and easy to be formed through common polymer morphing processes.

In this direction, polyhydroxyalkanoates (PHAs) biopolyesters are probably the only group of polymeric materials that fulfill all the aforementioned criteria. PHAs are biocompatible and biodegradable, while they exhibit enhanced barrier properties. These biopolymers are also compatible with various materials, such as other polymers, organic or inorganic nanoparticles, antimicrobial agents, etc. and consequently, they can be extensively modified towards the synthesis of novel smart, active and functional food packaging materials.

This chapter is focused on PHA applications in food packaging and food contact. Recent advances in this area, by means of peer-reviewed literature and patents, are going to be introduced and discussed. Additionally, innovative strategies towards the synthesis

of novel polymer blends, adequate for food contact applications, will be presented. Moreover, all the recent technologies and processes for the synthesis of functional surfaces based on functional PHAs, nanocomposites of PHAs and blends of PHAs with other polymers will also be reported.

Keywords: polyhydroxyalkanoates, biopolymers, biopolyesters, food packaging

1. INTRODUCTION

Food packaging is considered a vital procedure in the food industry. Its objectives include the: a) retainment of mechanical support, b) secure transition, c) shelf-life extension, and d) food preservation. Petroleum-derived polymers have been materials of significant importance in the food packaging applications due to their facile processing, low cost, and extensive mechanical properties [1].

The food-packaging materials provide specific protection to each type of food against oxygen, moisture, light, gases, and water vapor, thus, ensuring the maintenance of the appropriate packing conditions and eventually securing the product quality from taste or color deviation, grease oxidation, microorganism development, or degradation of nutrients [2].

The conventional food-packaging polymers are: i) polyethylene (PE), ii) polyethylene terephthalate (PET), iii) polypropylene (PP), iv) polyvinylchloride (PVC),v) polystyrene (PS), vi) polyamide (PA), and vii) laminated or co-extruded multilayer plastic films of ethylene-vinyl alcohol copolymers (EVOH) due to its potent oxygen and gas barrier properties [2, 3]. Indicative examples of food-packaging applications of the aforementioned polymers are presented in Table 1.

The environmental impact, the cost, and the migration of noxious ingredients into food matrix are the three basic issues, which render a polymer ideal for utilization, as a food-packaging material [4]. However, the majority of the currently available food packaging biopolymers do not satisfy the basic requirements of a packaging material regarding the barrier properties, in comparison with the conventional synthetic polymers.

The production of polymers is one of the largest and progressively developing manufacturing industries. The recycling industries aim at the reduction and the reuse of the polymers produced each year. However, the lack of cohesive recycling programs and the inability of the recycled materials to comply with the necessary criteria for food packaging render plastic recycling an expensive and time-consuming process [5, 6].

The food industry spends more than $110 billion per year on food processing and marketing [7], while10% of the total cost is spent on food packaging. Indicatively, at present, 50% of the total food packaging sales in the United States are covered by beverage and food packaging. The goals of achieving simplified food preparation, limited

demand for animal protein, improved material bioconversion and industrial processing, have led to the development of innovative food and beverage packaging trends, technologies and designs [7].

Plasticizers, fillers, and stabilizers are the most indicative packaging materials. Plasticizers enhance ductility, toughness, and flexibility of polymers, and reduce stiffness and hardness [8]. Fillers possess stable mechanical and barrier properties, while integration of cheap materials within the polymer body reduces the cost. Stabilizers inhibit the impairment of mechanical properties due to oxygenation and UV light [9].

Arguably, the main reasons for the utilization of bio-engineered polymer sources for packaging materials with improved sustainability are: (a) the constantly declining reserves of fossil fuels as the main source of synthetic packaging materials, (b) the environmental pollution caused by the disposal of non-biodegradable substances, through the process of landfilling, and (c) the conversion of fossil-fuel resources to carbon dioxide emissions, through the incineration of petrochemical carbon in resins and plastics [10, 11].

Additionally, the: a) potential harmful interactions of reused plastics with food, b) slow degradation rates of polymers and c) necessity for renewable sources have increased the global interest for biodegradable plastics, especially in food packaging and recycling applications [1, 12, 13].

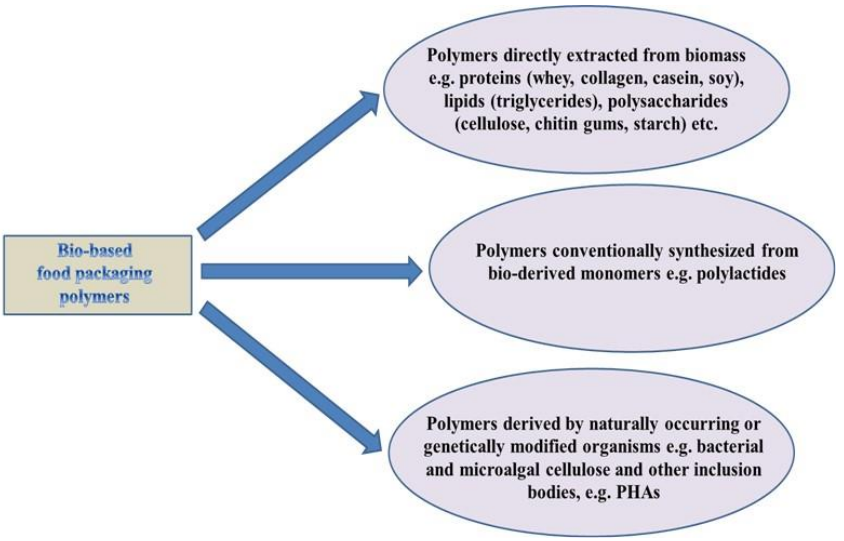
2. BIO-BASED FOOD PACKAGING POLYMERS

The adequate bio-polymers for food packaging applications are classified into three main classes as depicted in Scheme 1:

Although the naturally occurring compounds are sufficiently biodegradable materials, they are expensive substances with weak mechanical properties [14, 15].

Table 1. Food packaging applications of conventional polymers

Food-packaging polymers	**Examples of food-packaging applications**
Polyethylene (PE)	cooking oil, milk, water containers
Polyethylene terephthalate (PET)	food, beverage, and other liquid containers
Polyvinylchloride (PVC)	yogurt, spice, ice tea, margarine containers
Polypropylene (PP)	
Polystyrene (PS)	eggs and mushroom containers
Polyamide (PA)	meat, cheese, and other perishable food containers
Ethylene-vinyl alcoholcopolymers (EVOH)	meat, baby food, wine, juice, water, ketchup, milk, cheese containers



Scheme 1. Classification of bio-polymers for food packaging applications [1].

2.1. Biodegradable Polymers Suitable for Food Packaging

Biodegradable polymeric materials from renewable resources including polycaprolactone (PCL), PHAs, and poly(lactic acid) (PLA), possess various exploitable properties including thermoplasticity and processability on conventional plastics processing machines, thereby rendering them useful for biomedical tools and devices, drug encapsulation and targeted delivery systems, and packaging applications [16].

PCL aliphatic polyester is as thermoplastic biodegradable polymer obtained by crude oil chemical conversion. It possesses enhanced oil, water, chlorine and solvent resistance, low melting point, glass transition temperature, and viscosity, and improved processability on the conventional melt blending processes [16].

PLA is a bio-active, biodegradable, thermoplastic and aliphatic polyester obtained from the ring-opening polymerization of L-lactic acid. It derives from the fermentation procedure of cornstarch, cassava roots, chips or sugarcane, and can become biodegraded by fungi and some bacterial strains, such as *Alcaligenes faecalis* [17]. Until today, PLA constitutes the most commercially available biopolyester as a food packaging biopolymer for short shelf-life products, including drinking salad and sundae cups, containers, lamination and overwrap films or blister packages [15].

Although incorporation of plasticizers within the PLA main body reduces stiffness, it causes a decrease in transparency and oxygen barrier. PLA, when compared to polyolefins and PET, presents appropriate processability [18], enhanced transparency [19], market availability [19], printability [20] and biodegradability in a compost system [21]. Additionally, it possesses excessively high rigidity, low thermal resistance, and high

permeability. On the contrary, PLA films are not considered suitable moieties for food packaging mainly due to their substandard barrier and mechanical properties [22]. An important parameter for the successful large-scale industrial PLA production is the retainment of sufficiently low thermal degradation rates during use and processing [23].

2.1.1. PHAs: An Overview

PHAs are a large group of linear polyesters, synthesized by a variety of microorganisms and they are considered as great alternatives to petroleum-based polymers because they are versatile, bio-based, biocompatible and biodegradable [24]. In order to make PHAs the perfect candidate polymeric material for food packaging applications, scientists have tested numerous combinations, blends and approximations in order to create polymeric materials with a) improved thermal stability, b) mechanical properties, c) enhanced water and gas barrier properties, d) antimicrobial properties, etc. [25].

PHAs are biopolymers which may serve as energy sources and carbon stores in various microorganisms, under the existence of trace elements (e.g., Ca, Mg, Fe) or growth–limiting macro-elements (e.g., O, N, P, S) and an excess supply of carbon sources [26, 27]. They are considered biocompatible, biodegradable, thermally processable and flexible, and can be utilized as packaging materials, films, coatings, boxes, foam materials, fibers, biofuels, drug delivery vehicles and medical implants [28]. Although the unmanageable properties and high cost of PHAs have limited their applicability, novel approaches of metabolic bioengineering have developed adequate bacterial strains for the recombinant production of PHA structures with improved thermal and mechanical behavior [26].

PHAs are classified into three groups: short *(scl*-PHAs*)*, medium (*mcl*-PHAs), and long chain length (*lcl*-PHAs), resulting from hydroxy fatty acids of 3–5, 6–14 and >15 carbon atoms, respectively. *scl*-PHAs are synthesized by various bacteria such as *Ralstonia eutropha* (or *Cupriavidus necator*) and *Alcaligenes latus*. Additionally, *mcl*-PHAs can be synthesized by *Pseudomonas putida*. Copolyesters of *scl*- and *mcl*-PHAscan be produced by *Aeromonas hydrophila* and *Thiococcus pfennigii* [26, 27].

2.1.2. PHB: An Overview

PHB (polyhydroxybutyrate) is the most well-known and exhaustively studied PHA. It is produced by a variety of bacteria (e.g., *Ralstonia eutrophus*, *Methylobacterium rhodesianum, Bacillus megaterium*) as a response to physiological stress conditions [29]. It behaves similarly to conventional plastics and can be spun into fibers, molded, extruded, converted to films and utilized as the basic material for the synthesis of *scl*-PHA and *mcl*-PHA heteropolymers. The first possess poor elasticity and highly brittle behavior, while the latter highly ductile and easily molded [30].

PHB possesses stiffness, high melting temperature, and crystallinity. Despite being insoluble in aqueous media, it is biodegradable, presents piezoelectric behavior, strong optical activity, and efficient light barrier properties compared to PLA [31]. Long storage periods at room temperature increasingly affect the PHB brittleness. The convenient processing of PHB relies on its isotactic nature and absence of extensive chain branching [32-34]. However, the decrease of its thermal stability, molar mass and viscosity in the molten state act as a deterrent in its widespread utilization [35-39].

PHB has been extensively fabricated into packaging materials and disposable products, mainly due to its enhanced rigidity, lower flexibility, deformation value, and better performance at higher temperatures compared to PP [13].

The production cost of PHB is associated with numerous factors including the type of substrate, the selected bacterial strain, the cultivation process, and the downstream processing.

The employment of low-cost substrates [40, 46], the development of functional bio-engineering, design [47, 48], modeling [49], effective recovery methods [50-53] and profitable marketing are important parameters of the efficient utilization of PHB as a food packaging material [13].

2.2. Enhancement Techniques of PHA Properties for Food Packaging

PHA surface modification through combination with inorganic materials, enzymes, and polymers or by incorporation of nano-particulate compounds into the main body of PHAs improves thermal stability, mechanical properties, temperature, and machine cycle time [54].

Incorporation of different nanocomposite biopolymers (e.g., PHB) may lead to improved structural characteristics and organizational properties. Additionally, embodiment of nucleating agents decreases melt and brittleness during processing [55].

2.2.1. PHA Copolymerization

The mechanical and structural properties of PHAs can be manipulated through the copolymerization procedure producing materials of specific monomer compositions and with variable properties, such as strong crystallinity and thermoplasticity (e.g., PHB) or elastomeric behavior (e.g., poly(3-hydroxy-octanoate) (PHO)). PHB copolymers with hydroxy-hexanoate (HHx) or hydroxy-valerate (HV) are indicative examples of biopolymers with a variety of industrial applications [56].

Addition of 3-hydroxybutanoic acid to 3-hydroxy-valeric acid enhances tensile strength, glass transition temperature (*T*g), water permeability, and melting point, but decreases the impact resistance [57, 59]. Poly(3-hydroxy-butyrate-co-3-hydro-xyvalerate) copolymer (PHBV), due to its flexibility and piezoelectric properties, is more functional

than PHB, and can be utilized for film packaging, blow molding (e.g., bottles), and as paper coating [60, 61]. Due to its low melting point, it possesses a wide melt-processing window [62-64]. More specifically, increasing rates of hydroxy-valerate enhance the impact strength and flexibility, and decrease tensile strength [25, 64, 65-67]. However, PHBHV is considered difficult at processing due to its slow crystallization rates, and low elongation-to-break [68, 69].

Copolymers of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx), with decreased rates of 3HHx constitute materials with sufficient mechanical properties for flexible film formation [70]. Research results on the production of a novel PHA (PHACOS) (poly(hydroxy-6-acetylthiohexanoate*co*-4-acetylthiobutanoate)) with variable compositions of a new monomer containing thioester moieties (6-acetylthiohexanoic acid) by *P. putida* KT2442, are also reported [71].

2.2.2. PHA Blending

The addition of nucleating agents to PHB or PHBV, promotes the formation of extremely small spherulites with improved mechanical properties. The: a) morphology, b) processing conditions, c) glass temperature transition, and d) crystallinity are important factors that define the blend properties retaining biodegradability [33, 34, 54]. PHB and PHBHV blends with starch or thermoplastic starch [72, 73], PCL [16, 74, 75], PLA [76] and polyvinyl alcohol (PVA) [77], are reported in the literature. Additional blends of PHB or PHBHV with chemically compatible compounds such as plasticizers including saccharin, glycerol, tributyrin or triacetin [78, 79], as well as processing lubricants including mono- and/or tri-stearate [33, 34] display improved plasticity and modified mechanical properties [80].

PHBHV was also blended with keratin, derived from poultry, through a melt compounding process. The fully renewable packaging material had enhanced water, limonene and oxygen permeability properties compared to pure PHBHV [81].

The melt blending process of PLA with a variety of biopolymers decreases the cost and improves the morphological and functional behavior during the processing technologies [14]. Indicatively, the melt blending of bulk PLA with PHB of high crystallinity, two biopolymers with similar melting temperatures, enhances PLA crystallinity, improves its thermal and barrier properties and maintains its biodegradability and transparency producing adequate food packaging materials [82-85].

Furthermore, two specific processes, a polymer blending extrusion and a three-layer co-extrusion have been applied for the blending of PLA and PHBV towards the formation of films with different structures and improved gas barrier properties. The final blends had improved barrier and mechanical properties in comparison to single PLA and the 3 layered PLA/PHBV films [86].

2.2.3. PHA Nanocomposites and Films

Systems like multilayer polymeric films are made of two or more combined polymer layers in order to achieve specific gas barrier, optical, thermal and mechanical properties for food packaging applications. Incorporation of nanocomposites and organic or inorganic nanofillers of certain geometries (e.g., fibers, flakes, spheres and particulates) within the main body of PHAs produces materials with variable properties adequate for food packaging applications [87]. The incorporation of nanofillers within the main body of a polymer efficiently modifies the: a) biodegradation rate, b) morphology, c) crystallization behavior, d) stability and e) barrier, thermal and mechanical properties.

The incorporation of montmorillonite (MMT) clay in various polymers, including PE, PVC nylon, and starch produces materials suitable for food and beverage packaging applications [7].

Additionally, nanocomposites of PHAs reinforced with multi-walled carbon nanotubes (MWCNTs), layered silicates, layered double hydroxides (LDHs), or cellulose nanowhiskers (CNWs) are also reported in the literature [88]. More indicatively, high energy ball milling was applied for the productions of nanocomposites of PHBV with bacterial cellulose nanowhiskers (BCNW). The addition of BCNW as nucleating agents altered, in a positive way, the crystallinity behavior of PHBHV. This addition also reduced the oxygen permeability of the nanocomposite [89].

The increase of nanofiller dispersion directly improves the properties of the corresponding nanocomposites. More indicatively, the functionalization of PLA-PHB blends with CNCs produces novel inexpensive biodegradable food packaging materials of high stiffness, and low density with remarkable mechanical perspectives as short-term food packaging industrial materials [14, 15, 90-92].

Furthermore, biodegradable PHBV multilayer systems have been developed by adding a variety of nanostructured interlayers with high barrier and adhesive properties. The interlayers consisted of electrospun whey protein, zein, and pullulan fibers. The final multilayer systems exhibited enhanced water vapor and oxygen permeability properties [1, 93].

Films formed by compression molding of commercially available PHAs, PHB and PHBV, were evaluated for their transport properties. The results have shown a great dependency of the barrier properties, from presence of additives, while water permeability was affected by the crystallinity degree of the polymers [94].

Whey and gelatin were combined with PHA toward the formation of multilayer films for the improvement of the optical, structural and mechanical properties of the later. The results have shown that the final application of the film directly affects the enhanced properties of an optimal film formulation, compared to pure PHA [95].

PHB films were prepared by electrospinning technique and were evaluated for their use as food packaging material. Post-processing of PHB electrospun fiber mats was performed in order to optimize their final physical properties. The thermal optical,

mechanical and barrier properties of the films were evaluated according to the annealing time and cooling method. Homogeneous and transparent films were obtained at an annealing temperature (160ºC) that is lower than PHB melting point, while slow cooling rates resulted in films with superior oxygen, limonene and water vapor permeability properties. The electrospun films had similar mechanical properties to those prepared by conventional compression molding, but they were tougher and with enhanced elongation at break [96].

Gamma irradiation has been applied on PHB/modified sepiolite (PHB/SP) nanocomposites, in order to improve their mechanical properties through a chain-scissions, crosslinking, unsaturation and decomposition process within the polymeric chains. The increased thermal stability and the biodegradability of the nanocomposites render them a possible alternative material for biodegradable food packaging [97].

The grafting process of sepiolite nanoclay onto poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-*co*-4HB)] biopolymer by a reactive extrusion, was implemented in the presence of epoxy-based styreneacrylic oligomer (ESAO). The newly formed sepiolite-grafted nanocomposite had improved thermal and mechanical properties, as the grafting of sepiolite enhanced the stiffness and thermal stability of P(3HB-*co*-4HB) without altering its toughness and ductility [98].

Silver nanoparticles (AgNP) are one of the most studied antimicrobial materials, mainly due to their broad spectrum of antimicrobial activity. Their uses are expanded from medical application all the way to food application, as they are compatible with many known materials. PHBV films that were made by deposition of electrospun PHBHV18/AgNP fiber mats, over PHBV films that were made by compression molding, were evaluated for their antiviral properties with very good results that show the suitability of PHBV18/AgNP electrospun coatings for antiviral surfaces [99]. However, it has been proved that ZnO and TiO_2 nanoparticles are safer and more suitable for food packaging applications [100].

Additionally, PHB and PHBV coatings loaded with antibiotics were applied on titanium implants by dip-coating technique [101].

Bio-based multilayered films with a PHBV8 film as a substrate were manufactured. Zein interlayers that contain cinnamaldehyde, were directly electrospun onto the PHBHV8. The films were characterized by their water vapor and oxygen permeability, intermolecular arrangement, thermal properties, and transparency. Cinnamaldehyde is a well-known terpenoid with antimicrobial properties. The films that contained cinnamaldehyde were tested for their antimicrobial activity against *Listeria monocytogenes* presenting positive results. The overall conclusion of this study was that the direct electrospinning of cinnamaldehyde loaded zein fibers, on a polymer substrate made by PHBV, is a method that can produce active bio-based food packaging systems [102].

Modified graphene oxide (GO) by alkylation with butyl-, octyl- and hexadecylamine, was melt blended with poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3,4HB)]. The nanocomposites were evaluated for their high degree of processability, biodegradability, and thermal stability, mechanical and rheological properties. The addition of graphene oxide enhanced the decomposition temperature of the polymer, proportionately to the alkyl chain length. The modification of the GO, also improved the compatibility of the later with the polymer. Finally, even though the overall thermal and mechanical properties of the polymer were improved, the addition of either modified and/or unmodified GO to the polymer induced a chain degradation during the melt extrusion process [103].

Natural vermiculite and organoclay were blended, via melt mixing, with PHBV, aiming at the improvement of the biopolymer thermal stability and mechanical properties [104].

PHBHHx films with antimicrobial activity were prepared by mixing the antibacterial peptide Nisin with PHBHHx, via a solvent casting technique. The antimicrobial efficacy of PHBHHx/Nisin films against *Micrococcus luteus* was studied and the results have shown that Nisin can be used for the production of antibacterial PHA for biomedical and food packaging applications [105].

CONCLUSION

The majority of the conventional food packaging polymers is considered non-degradable–and produced either in part or fully from renewable resources. Packaging waste constitutes a considerable part of municipal waste inducing serious environmental concerns. Additionally, plastic recycling is not considered a practical and economical solution due to the resulting contamination of the difficult-to-recycle plastics. Shifting towards biodegradable materials for food packaging applications, suitable for composting or recycling, with negligible permeability to gases, humidity, and odors, and improved dimensional stability, brilliant appearance, and opacity is a key factor for the production of functional plastics ideal for food packaging.

Despite their relatively high cost of production compared to petrochemical products, bioengineered PHAs and PHA blends of mixed cultures from a variety of waste streams represent an effective solution in the production of functional materials applicable in the food industry. The insufficient flexibility of PHAs may be confronted by the blending procedure, thus reducing the intractable brittleness. Incorporation of nanofillers within the main body of PHAs results in higher Young’s modulus, enhanced toughness and deterioration of PHA biodegradation rates, in combination with reduced water permeability and improved antimicrobial efficiency.

Another important issue that has to be addressed is the limitation of the contingent migration of the PHA degradation products or incorporated nanoparticles to food, mainly during the biodegradation or processing of the corresponding packaging materials. New efficient strategies for the development of optimized gas-barrier function in combination with the design of inexpensive scaled-up bioprocesses and production rates are currently under investigation. Furthermore, research results on the incorporation of active antimicrobial, oxygen-scavenging or antioxidant nanocomposite compounds into the main body of the packaging materials indicate an alternative and efficient strategy towards the preservation and prolongation of the microbiological shelf life of perishable foods during the refrigerated storage.

The development of nanocomposite packaging technologies in order to achieve a combination of active performance and physical amplification represents the main future research directive. The correlation analysis of the relationship between PHA biosynthesis and metabolism through enzymatic and genetic studies will contribute to the achievement of this goal.

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