POLYHYDROYALKANOATES: FROM BASIC RESEARCH AND MOLECULAR BIOLOGY TO APPLICATION

AMRO ABD AL FATTAH AMARA

Genetic Engineering and Biotechnology Research Institute,
Mubarak City for Scientific Research and Technological Applications. Egypt.

e-mail: amroamara@web.de

ABSTRACT: This review describes the Polyhydroxyalkanoate (PHA), an intracellular biodegradable microbial polymer. PHAs is formed from different types of three hydroxyalkanoic acids monomers, each unit forms an ester bond with the hydroxyl group of the other one and the hydroxyl substituted carbon has *R* configuration. The C-3 atom in β position is branched with at least one carbon atom in the form of methyl group (C1) to thirteen carbons in the form of tridecyl (C13). This alkyl side chain is not necessarily saturated. PHAs are biosynthesized through regulated pathways by specific enzymes. PHAs are accumulated in bacterial cells from soluble to insoluble form as storage materials inside the inclusion bodies during unbalanced nutrition or to save organisms from reducing equivalents. PHAs are converted again to soluble components by PHAs depolymerases and the degraded materials enter various metabolic pathways. Until now, four classes of enzymes responsible for PHAs polymerization are known. PHAs were well studied regarding their promising applications, physical, chemical and biological properties. PHAs are biodegradable, biocompatible, have good material properties, renewable and can be used in many applications. The most limiting factor in PHAs commercialization is their high cost compared to the petroleum plastics. This review highlights the new knowledge and that established by the pioneers in this field as well as the factors, which affect PHAs commercialization.

KEYWORDS **PhaC synthase, Polyhydroxyalkanoate, Polyhydroxybutyrate, Plastic, Application, commercialization**

1. INTRODUCTION

1.1 Polymers

Polymers are naturally occurring or synthetic compounds, consisting of large molecules, made up of linked series of repeated monomers joined by chemical bonds through chemical reactions (polymerization, polycondensation, polyaddition) to form the polymer. Plastic is a generic name of synthetic, semi-synthetic or natural materials that can be moulded or extruded into objects, films, filaments or used to make, for example coatings and adhesives. It is mainly derived from petroleum oil.

1.2 Plastic Problems

The manufacturing of plastic has increased significantly since the 1940s and has successfully replaced wood, mud, metals, glass and other materials [1, 2, 3, 4]. The low cost, stability, durability, good mechanical and thermal properties of plastic make it the best choice for widespread applications [5]. The extensive use of materials made from plastics causes a worldwide problem because they are non-degradable [3, 5, 6, 7].

Plastic packages take up about 1/3 of the volume of municipal wastes because of their low density [8, 9, 10]. Plastics consistently make up 60 to 80% of the marine debris [11]. Barnes (2002) accounts the amount of debris around the coastline of the UK has doubled between 1994 and 1998, in parts of the Southern Ocean it increased 100-fold [12]. General data show that world production of packages is on the level of about 1500 million tons/year [10]. Three hundred and sixty thousand tons of plastic for the production of bottles have been manufactured in Europe in 2002 and the world currently needs around 140 million tons of plastic yearly [13]. Processing these plastics needed nearly 150 million tons of fossil fuels, which with their high treatment cost and the emission of toxic compounds like dioxin during plastic incineration increase the gravity of the problem [14].

1.3 Biopolymers

Steinbüshel has classified the biopolymers based on their chemical structure into eight classes: i) nucleic acids, ii) "polyamides" such as a protein poly-(amino acids), iii) polysaccharides, iv) organic polyoxoesters such as poly(hydroxyalkanoic acids), poly(malic acid) and cutin, v) polythioesters, iv) inorganic polyesters with polyphosphate, vii) polyisoprenoides such as natural rubber or Gutta-Percha and viii) polyphenols such as lignin or humic acids [15]. Not all biopolymers have the same properties, few species can be used as plastics and not all of them are biodegradable like polythioesters [16 - 18].

Fig. 1: Bacterial cells producing a PHA polymer (white granules inside the cell)

The most widely known bioplastics, which are produced by microbes, are polyhydroxyalkanoates (PHAs) and their derivatives [19-21]. PHAs can solve the solid waste problem by substituting petroleum plastic [4, 22]. PHAs have proved to be similar in material properties to petroleum plastics, while PHAs are produced from renewable resources and are biodegradable [22, 23].

1.4 History of PHAs

PHAs are widely distributed within different microbial species and are accumulated intracellularly in the form of storage granules. Perhaps, the first report about lucent granules of PHAs in bacterial cells (Fig. 1) was made by Beijerinck in 1888 (reported in Chowdhury, 1963) [24]. The French scientist Maurice Lemoigne, who worked at the Lille branch of the Pasteur Institute - France, had the interest to characterize these inclusion bodies, which were found in *Bacillus spp*.

The work done by Lemoigne and co-workers were published in 27 publications from 1923 until 1951 [25]. Lemoigne was the first to report that the bacterial granules components (Lemoigne describes it also as reserved material) were not ether soluble, as in lipids, and later reported that polyhydroxybutanoate (PHB) is the major constituent of these granules. Using microscopic observations, saponification numbers, and autolysis whose solubility and melting point (T_m) variation were correlated with molecular size proposed a polyester structure with formula $(C_4H_6O_2)_n$. Lemoigne has also reported that PHB could cast into film like cellulose nitrate material [25 - 29].

During the following years, interest in this unknown material was negligible. In 1953 Weibull had isolated the granules of *Bacillus megaterium* by dissolution of the cell wall with a lysozyme [30]. The first report on functions of PHB appeared in 1958 by Wilkinson and Wilkinson who obtained morphologically intact granules from *Bacillus cereus* using alkaline hypochlorite, and Macrae and Wilkinson in 1958 who described poly-*β*-hyroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium* [31, 32]. Doudoroff and Stanier (1959) and Stainer et al., (1959) found that PHB can be produced by photosynthetic assimilation of organic compounds by phototrophic bacteria and they described that the reaction involved in the metabolic pathway is responsible for the biosynthesis of PHB from acetic acid [33, 34]. Merrick and Doudoroff (1961) isolated and described native PHB granules of hemoheterotropic bacteria, *Rhodospirillum rubrum* and *B. megaterium* [35]. In 1966, Lusty and Doudoroff have shown a study on depolymerases which were able to hydrolyse PHB [36]. Lundgren et al., (1965) showed that only the cells that are extracted with chloroform could yield high molecular weight PHB [37]. Schlegal at the University of Göttingen, Germany in 1961 observed that *Cupriavidus necator* (formally *Wautersia eutropha*, *Ralstonia eutropha* and *Alcaligenes eutrophus*), could accumulate very large amount of PHB in a media with low nitrogen content [38]. Merrick and Doudoroff (1964) were the first to identify the enzyme responsible for PHB polymerization which is the synthase or the polymerase as was known at that time followed by work done by Griebel et al., (1968) [39, 40]. Schlegal working with *C. necator* and Dawes working with *Azotobacter berjerinckii* (University of Hull, England) in 1973 simultaneously were the first to isolate and characterize the enzymes, which catalyse the PHB monomer synthesis [41, 42].

Wallen gave the first indication that the polymer discovered by Lemoigne may contain proportions of 3-hydroxyacids other than 3-hydroxybutyrate and Rohwedder, who, in 1974, reported heteropolymers of 3-hydroxybutyrate and 3-hydroxyvalerate in chloroform extracts of activated sewage sludge as major constituents with C6 and possibly C7 3-hydroxyacids as minor components [43]. This heteropolymer had a lower melting point than PHB and, unlike the homopolymer, was soluble in hot ethanol. Findlay and White in

1983, using GC analysis, detected at least 11 short-chain 3-hydroxyacids, the principal ones being 3HB and 3HV [44].

De Smet et al. characterized PHA_{MCL} in *Pseudomonas oleovorans* during growth on octane [45]. Huisman et al. confirmed that PHA_{MCL} accumulation was the common feature of fluorescent pseudomonads [46]. PHAs are also produced by gram-positive, gram-negative bacteria and phototrophic bacteria as well as archaea [47 - 52].

PhaC synthase from an extreme halophilic archaeon has been enzymologically and biochemically characterized [53, 54]. The first halobacterial PHAs synthase gene sequenced is from *Haloarcula marismortui* [55]. Another putative polyester synthase gene was identified in the genome of an uncultivated archaebacterium belonging to *Crenarchaeota* [56].

2. PHAs NOMENCLATURE

There are many ways to abbreviate PHAs species but two of them are the most dominant; they are based on the number of their carbon atom or classifying them to Short, Medium or Long Chain Length. PHAs created from three to five carbon atoms are called Short-Chain-Length, termed SCL, while PHAs with six to fourteen carbon atoms are called Medium-Chain-Length, and termed MCL [48]. In fact, even this abbreviation system is based on the number of C atoms in their monomeric unit's composition but at the same time it reflects the different physical properties where SCL PHAs are solid or semisolid while MCL PHAs are latex like, rubber or elastomers. The PHAs which contain more than fourteen carbon atom are called Long-Chain-Length (LCL). Genes coding for proteins involved in the biosynthesis of PHA are referred in alphabetical order as *phaA* (*β*-ketothiolase), *phaB* (acetoacetyl-Co A reductase), *phaC* (PHA synthase), *phaG* (3-hydroxyacyl-acyl carrier protein-Co A transacylase), etc. [48, 57].

The names of genes involved in PHAs degradation are referred to opposite alphabetical order such as *phaZ* for PHA depolymereses, *phaY*, *phaX*, *phaW* etc. The genes for phasins and regulator proteins are referred to as *phaP* and *phaR*, respectively. The origin of the gene or protein can be also referred to the first letter of the gene and the species as subscript e.g., $phaC_{Pa}$ refers to *Pseudomonas aeruginosa*. It is important to sign that using the numbering style to differentiate between the monomer structures is preferable when copolymer is present such as P(3HB-*co*-4HB). If the polymer is well known it is preferable to use their abbreviation instead of using SCL/MCL style. For example, polyhydroxybutyrate usually abbreviate as PHB or P3(HB) and polyhydroxybutyrate-*co*-polyhydroxyvalirate abbreviate as PHB-*co*-PHV or P(3HB-*co*-3HV). Alternatively, in case of MCL PHAs and because of the big number of their monomeric composition PHA_{MCL} is usually used. Finally, PHAs could be abbreviated using any of the described styles based on the situation. An example for the different abbreviation styles for polyhydroxybutyrate-*co*-polyhydroxyvalerate are PHB-*co*-PHV, P3(HB-*co*-HV), P(3HB-*co*-3HV), P(3-*β*HB-*co*-3-*β*HV), P3*β*(HB-*co*-HV). Few authors are still using *β* in PHA abbreviation. PHAs will refer to different kinds of polyhydroxyalkanoates including SCL, MSL and LCL.

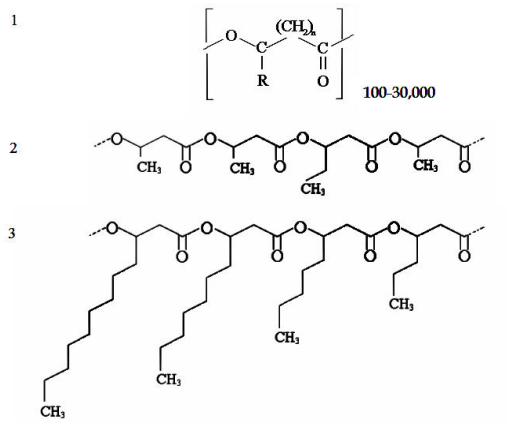

Fig. 2: Chemical structures of PHAs.
1) General structural of PHAs. 2) The copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV) as example of SCL. 3) P(HHX-*co*-HO-*co*-HD-*co*-HDD-*co*-…..) as an example for MCL.

3. PHAs CHEMICAL STRUCTURE

Like any polymer, PHAs are chains of monomers repetition either homopolymer or heteropolymer. In general, PHAs known to be linear, are composed of 3-hydroxy alkanoic acid monomers units, each unit form an ester bond with the hydroxyl group of the other one. The hydroxyl-substituted carbon atom has *R* configuration in all characterized PHAs. At the C-3 atom (β position), an alkyl group which length can vary from methyl (C1) to tridecyl (C13) is located as in Fig. 2 [48]. This alkyl side chain is not necessarily saturated. Unsaturated, aromatic, epoxidized, halogenated, and branched monomers have been reported as well [21, 58 - 66]. By cross-linking of unsaturated bonds substations in the side chains of PHAs, for instance, can be added chemically [67].

Lütke-Eversloh et al was the first report to produce biopolymers with thioester linkages in the polymer backbone using *C. necator* in media containing 3-mercaptopropionate (3MP) or 3-mercaptobutyrate (3MB) in addition to 3-hydroxybutyrate as constituents [16, 17].

Many factors affect the PHAs chemical composition like the microbial strain, the substrate, the cultivation condition, the extraction method, the number of *phaC*, *phaB* genes, the regulator *phaP* (phasin) and the presence of inhibitors. They inhibit different pathways especially those, which supply the synthases with different kinds of monomer or inhibit other pathways, which consume these monomers for their own or degrade it to shorter units like β oxidation pathway.

The PHA_{MCL} is synthesized with an average molar mass ranging from 5×10^4 to 2×10^5 g/mol, whereas PHB can be produced with an average molar mass of $>1 \times 10^6$ g/mol. In general, the PHA composition depends on the PHA synthases, the carbon source and the metabolic routes involved [19, 68].

PHAs' molecular weights have been established by light scattering, gel permeation chromatograph and sedimentation analysis. Their monomer composition has been

determined by gas chromatography (GC), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) analysis [16, 17, 44].

4. PHAs PHYSICAL PROPERTIES

PHAs' crystal structure, polydispersities, melting point, enthalpy of fusion, glass transition temperature and mechanical properties were established by the use of different procedures. PHAs, ranging from stiff, brittle to rubber-like make them a close substitute for the synthetic plastic. PHB, compared with plastic like polypropylene, is considered as having the most common properties [69], and PHB was in fact a thermoplastic [70] as presented in Table 1.

In the case of PHB-within the cell it exists in a fluid, amorphous state. However, after extraction from the cell with organic solvents, PHB becomes crystalline and rigid but brittle material [71]. The brittleness of PHB during mechanical processes makes it unresisting to stress. The major problem of PHB is that it decomposes near its melting point. However, if there is another monomer included in the structure of PHB such as 3HV it decreases PHB crystallinity and increases its elasticity [72].

The rubber or latex-like PHA_{MCL} exhibits physical properties significantly different from the PHA_{SCL} with respect to the melting temperature and the extension at the break point as in Table 1.

Table 1: Properties of some PHAs and polypropylene

Parameter	P(3HB)	P(3HB-*co*-3HV)	P(3HB-*co*-4HB)	P(3HO-*co*-3HH)	PP
T_m (°C)	177	145	150	61	176
T_g (°C)	2	-1	-7	-36	-10
Crystallinity (%)	70	56	45	30	60
Extension at break (%)	5	50	444	300	400

T_m, melting temperature; T_g, glass transition temperature; P(3HB), poly(3-hydroxybutyrate); P(3HB-*co*-3HV), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) containing 20% 3HV; P(3HB-*co*-4HB), poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) containing 16% 4HB; P(3HO-*co*-3HH), poly(3-hydroxyoctanoate-*co*-3-hydroxyhexanoate) containing 11% 3HH, and PP is polypropylene (Doi, 1990).

5. LIMITATIONS OF PHAs COMMERCIALIZATION

The production cost of PHAs is the main commercialization-limiting factor for these biopolymers. PHAs' accumulation requires special growth conditions and it's usually imbalanced nutrition that causes slow growth.

The Imperial Chemical Industries in Billingham, United Kingdom produced copolymers of PHB-co-PHV on a large scale [69]. The market penetration is rather scarce,

and the products are known as "Biopol" trademark. In 1990, the German company Wella used Biopol-made flasks for a new shampoo. Bacteria-produced PHA is approximately, five to ten times more expensive than its competitors, polypropylene or polyethylene. Although this natural product is promising, its price at that time was $17 to $22/kg [73]. The market price of Biopol prepared by ZENECA BioProducts, a subsidiary company of ICI, was about $16/kg. Development of better fermentation and purification technologies and the use of genetic engineering lowered the price, which becomes $4/kg, but it is still expensive compared to its competitors.

Major expenses in the production of PHA are determined by the cost of the fermentation substrates, and the extraction of the polymer from the cells [69]. Another trill for PHAs commercialization was started in 1996, when the American company Monsanto bought the Biopol material from ZENECA BioProducts. Monsanto tried to produce Biopol by using transgenic plants. The genes originating from bacteria were transferred to plants by genetic engineering methods, which produce the polymer. Monsanto was optimistic about the PHAs commercialization researches especially those concerning the transgenic soya and oilseed rape. Unfortunately, Monsanto has terminated its project in late 1998. Developing countries did not have enough experience in the field, but had other positive factors to overcome PHAs production cost like cheap raw material resources and available work forces [74].

Yamane et al. reported that the major cost in PHA production is their substrate [75]. It is important to highlight that if microbial cells in their optimum condition are able to accumulate PHA it will not exceed certain limit based on their size capacity. Unfortunately, usually big microbial cells have lower generation time. The cheapest substrate cost is $0.22/kg of PHA while the cost of polypropylene is $0.185/kg [76]. The substrate cost affects the overall cost but the cheapest substrate is not always the ideal choice. When the PHB productivity increased from 1.98 to 3.2 g/h, the PHB production cost decreased from $5.37/kg to $4.91/kg [77]. In a laboratory fed-batch system using *A. latus*, the highest reported productivity was 4.94 g/h with cost about $2.6/kg [77].

Amara and Salem were describing phenol degradation using microbial strains have the PHA production system which could be used for PHAs production during phenol degradation process [78].

5.1 Why are PHAs Still so Attractive?

Scientists and industrialists have been looking for a replacement of petroleum plastic, which causes a lot of environmental, health, biodiversity and economic problems with a more safe type of plastics, which are bio-friendly. PHB, and other member of PHAs family provide the hope as a solution to those problems with petroleum plastic. PHAs are biodegradable without any toxicity, bio-evaluable and to some extent do not make any stimulation to the human immune system even if the human body can degrade the polymer after a period of time. This will match the request of many medicinal and pharmaceutical applications.

6. PHAs APPLICATIONS

6.1 Industrial Applications

The first potential application of PHAs polymers was recognized in the 1960s [79]. PHAs patents cover a wide range of PHAs products such as coating and packaging, bottles, cosmetic containers, golf tees, and pens [80 - 82]. PHB alone or with 3HV as a copolymer have been used as diaper back sheets and films [83, 84]. PHAs have also been processed into fibers, for a nonwoven fabrics material [85]. PHB and PHB-*co*-PHV have been used as hot-melt adhesives [86]. PHAs are used as developer compositions and toner or in ion-conducting polymers [87, 88]. PHAs are used as latex in paper-coating applications, [89], in dairy cream substitutes [90], in food flavour delivery agents [91], or as raw materials for the synthesis of enantiomerically pure chemicals and paints [23, 92 – 96]. PHAs are also used in packaging materials or agricultural foil [97]. To produce a coating, the PHA latex is sprayed onto a substrate such as paper. After the evaporation of water, the PHA latex particles readily coalesce into a film [67]. PHA might also find marine applications, such as in fishing nets [98, 99].

Aiming to widen the range of industrial applications and establishing a method for changing PHAs compositions as well as PHAs' overproduction Amara et al., described a new simple strategy for *in vivo* random mutagenesis and mutants selection using synthase gene from *Aeromonas punctata* employing the mutator strain *Escherichia coli* XL1-Red and Nile red plates. The mutants mediated synthesis of PHAs with an increased weight average molar mass. Mean while the molar percentage is different which an early indication about the possibility of changing the substrate specificity using random mutagenesis. *In vivo* random mutagenesis proved to be a versatile tool to isolate mutants exerting improved properties with respect to PHA biosynthesis [100, 101].

Taguchi et al., used another strategy based on *in vitro* mutagenesis for $phaC_{Re}$ using PCR [102, 103].

6.2 Biomedical Applications

Due to the relatively high cost of PHAs production, it's wise to apply PHAs for some cost effective applications like medicinal instruments.

PHAs have been proved biocompatible in tissue engineering, implantations, etc. Many prokaryotic and eukaryotic organisms are able to produce low molecular weight PHB molecules that are complexed with other biomolecules such as polyphosphates and that are present at low concentrations [104].

Over the past years, PHAs were used to develop many devices and material useful for clinical purposes such as sutures fasteners, meniscus repair devices, rivets, tacks, staples, screws, (including interference screws), bone plates and bone plating systems, surgical mesh, repair patches, orthopedic pins (including bone filling augmentation material), adhesion barriers, stents, guided tissue repair regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, pericardial patches, bulking and filling agents, vein valves, bone marrow scaffolds, meniscus regeneration devices, ligament,

tendon grafts, ocular cell implants, spinal fusion cages, skin substitutes, dural substitutes, bone graft substitutes, bone dowels, wound dressing and hemostats [105 - 111].

To assess the biocompatibility of PHB, the structural organization of cellular molecules involved in adhesion was studied using osteoblastic and epithelial cell lines. On PHB, both cell lines revealed a rounded cell shape due to reduced spreading. The filamentous organization of the actin cytoskeleton was impaired. In double immunofluorescence, analyses the co-localization of the fibronectin with the fibril actin was demonstrated [112]. The investigated properties of PHB and PHB-*co*-PHV films proved to be fundamentally similar [113 - 117]. PHB-*co*-PHV film was chosen as a temporary substrate for growing retinal pigment epithelium cells as an organized monolayer before their subretinal transplantation. The surface of the PHB-*co*-PHV film was rendered hydrophilic by oxygen plasma treatment to increase the reattachment of D407 cells on the film surface. The cells were also grown to confluency as an organized monolayer suggesting PHB-*co*-PHV film as a potential temporary substrate for subretinal transplantation to replace diseased or damaged retinal pigment epithelium [118].

Tesema et al. and Malm et al. implanted PHB nonwoven patches as transannular patches into the right ventricular outflow tract and pulmonary artery in 13 weanling sheep [119 - 122]. It is concluded that PHB nonwoven patches can be used as a scaffold for tissue regeneration in low-pressure systems. The regenerated vessel had structural and biochemical qualities in common with the native pulmonary artery [122]. PHAs have been used in tissue engineering, as antibiotic carrier, and many other medicinal applications [123, 124, 125].

Chen and Wu recently reported that PHAs possesses the biodegradability, biocompatibility and thermo-processibility for not only implant applications but also controlled drug release uses. PHAs show a promising future in pharmaceutical application such as drug delivery, which open a new approach. The many possibilities to tailor-made PHAs for medical implant applications have shown that this class of materials has a bright future as tissue engineering materials [126].

7. PHAs DETECTION

7.1 Detection Using Stains

Sudan black, Nile blue and Nile red can be easily used for PHAs detection [74, 127, 128, 129]. Nile red is the most common stain used for screening of PHA accumulating bacteria [129]. Amara et al, described a very simple method using Nile red, it depends on the appearance of a clear red color if the colonies on plates are incubated at -4 ^{o}C for appropriate time [74] as illustrated in Fig. 3 and 4.

Fig. 3: Agar plate shows fluorescent colonies, which indicate the presence of PHA after visualization by UV at 312 nm [74].

Fig. 4: Nile red plate after incubation at -4 ^{o}C. The red colonies shows the +ve PHA accumulation while the white colonies show –ve PHA accumulation [74].

7.2 Quantitative Analysis of PHA using GC/MS

One simple method has been described by Brandl et al. using GC (Gas Chromatography) to analyze various PHAs and further described in detail by Timm and Steinbüchel, [130, 131]. The amount of PHAs should be calculated based on their different monomeric compositions, eg. if 3HB and 3HHx (3-hydroxy-hexanoate) have the same area the amount of PHB monomer will be bigger because it has the lower molecular weight.

8. BIOCHEMICAL ASPECT ABOUT PHAs BIOSYNTHESIS

8.1 PHA Synthases

PHA synthases are the key enzymes of PHA biosynthesis. PHA synthases use coenzyme A thioesters of hydroxyalkanoic acids (HA) as substrates and catalyze the polymerization of HAs into PHA with the concomitant release of CoA. Twenty years ago in three different laboratories, PHA synthase operon of *C. necator* has been cloned [132, 133, 134].

8.2 PHA_{SCL} Biosynthesis

PHB is the most extensively investigated PHAs. The PHB biosynthesis pathway consists of three distinct enzymatic reactions. Examination of the enzymes leading to the formation of PHB has shown that the regulation of PHB synthesis is controlled by acetyl-coenzymes A (CoA) acyltransferase [135, 136]. PHB is synthesized from acetyl coenzyme-A (acetyl-CoA) by a sequence of three enzymatic reactions. Synthesis of PHB is believed to be controlled by the first enzyme, 3-ketothiolase that is inhibited by coenzyme A (CoASH) [137].

(i) *β*-ketoacyl-CoA thiolase (encoded by *phaA*)

The first reaction comprises the condensation of two acetyl coenzyme A molecules into acetoacetyl-CoA by *β*-ketoacyl-CoA thiolase (*β*-ketothiolase).

(ii) NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by *phaB*)

The second reaction is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase.

(iii) PHB synthase (encoded by *phaC*)

Finally, the (*R*)-3-hydroxybutyryl-CoA monomers are polymerized to PHB by PHB synthase.

Under balanced growth conditions, acetyl-CoA is fed into the tricarboxylic acid cycle and the resultant CoA inhibits acetyl-CoA acyltransferase and PHB synthesis. Under nutrient limitation (e.g., Oxygen limitation) and carbon excess, NADH oxidase activity decreases, NADH increases and inhibits citrate synthase and isocitrate dehydrogenase, and acetyl-CoA acyltransferase by CoA is overcome. The ensuing condensation reaction forms acetoacetyl-CoA and initiates PHB synthesis [135, 136]. The high NADH/NAD ratio caused oxygen limitation rapidly readjusts as PHB synthesis starts, and PHB assumes the role of an alternative electron acceptor [135].

The degradation of PHB is initiated by PHB depolymerase to form D(-)hydroxybutyric acid. An NAD-specific dehydrogenase oxidizes the acid to acetoacetate, which is an intermediate common to the biosynthesis and degradation of PHB [99].

8.3 PHA_{MCL} Biosynthesis

8.3.1 PHA_{MCL} Biosynthesis from Related Substrates

In 1983, De Smet described the accumulation of PHA, the PHA_{MCL}, in *Pseudomonas oleovorans* grown on octane as sole carbon source [45]. The compositions of the PHAs formed by pseudomonads of the rRNA homology group I were directly related to the structure of the alkane, alkene or fatty acid used as carbon source [46, 130]. These substrates are referred to as ‘related substrates’. When the carbon source consists of fatty acids with 6 to 12 carbon atoms, monomers of the PHA are of the same length as the carbon source or are shortened by 2, 4 or 6 carbon atoms.

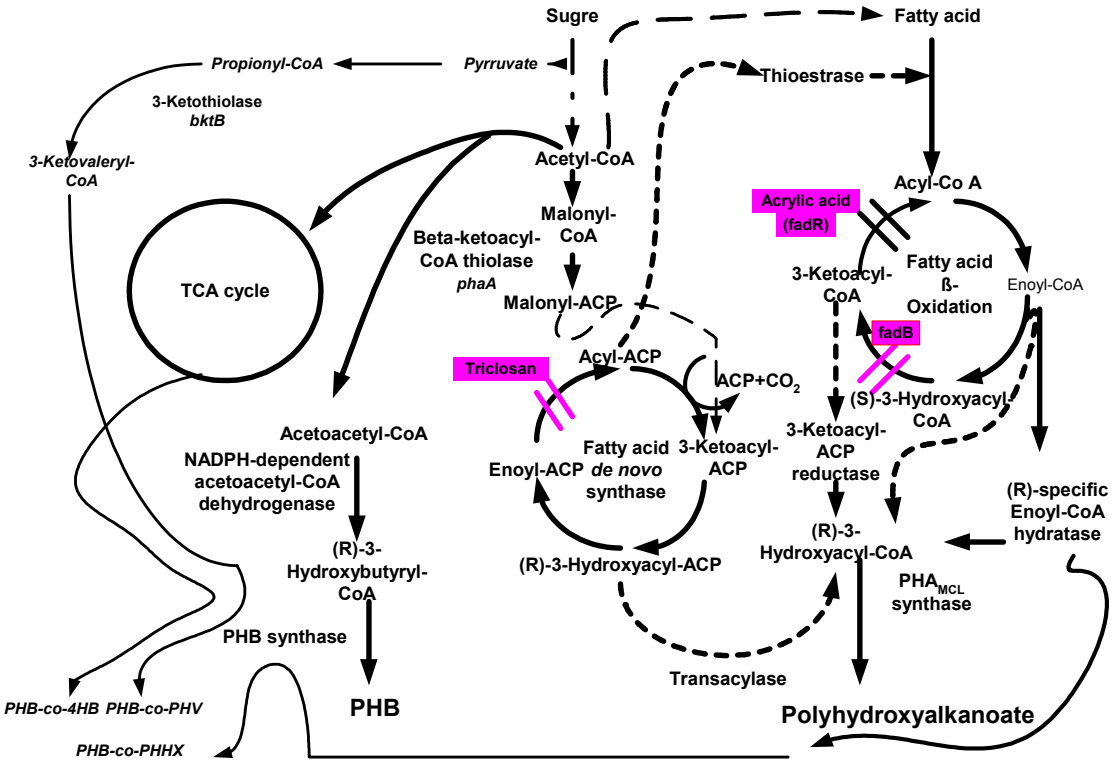

Fig. 5: Metabolic pathways leading to the biosynthesis of PHAs, PHB, PHB-*co*-PHV, -*co*-4HB, -*co*-PHX, PHA_{MCL}. Acrylic acid is the inhibitor of fatty acid *β*-oxidation pathway and triclosane is the inhibitor of fatty acid *de novo* biosynthesis pathway.

Aeromonas punctata produces a random copolymer of 3HB and 3HHX, when growing on even-numbered fatty acids or olive oil as sole carbon source. PHA production in *A. punctata* proceeds from the *β*-oxidation pathway intermediate 3-hydroxyenoyl-CoA, which is hydroxylated by the enantiospecific enoyl-CoA hydratase (Fig. 5).

8.3.2 PHA_{MCL} Biosynthesis from Unrelated Substrates

Synthesis of PHAs other than PHB which are not related to their growth substrates occur relatively rarely in Nature [22]. These substrates, which do not resemble the monomers of the accumulated polymer, are named ‘’unrelated substrates’’.

The PHAs production under unbalanced conditions is highly naturally engineered. The first indication about the ability of synthases to utilize products from other pathways in their monomeric composition was reported by Wallen and Rohwedder in 1974 who, reported heteropolymers of HB and 3-hydroxyvalerate in chloroform extracts of activated sewage sludge as major constituents with C6 and possibly C7 3-hydroxyacids as minor components [43]. Slater et al., identified that a second *β*-ketothiolase gene in *C. necator* is responsible for synthesis of 3-ketovaleryl-CoA [138].

Pseudomonads of the rRNA homology group I like *P. putida* KT2442, accumulate PHAs that consist primarily of C10 and C8 monomers, when grown on sugars or gluconate [131, 139]. These monomers are derived from intermediates of fatty acid biosynthesis and the composition of the PHAs is a reflection of the pool of fatty acid biosynthetic intermediates.

8.3.3 Metabolic Engineering of PHA_{MCL}

8.3.3.1 Linking Metabolic Pathways

Different PHAs could be produced from simple cheap carbon sources; alternatively, the carbon source may be derived from waste materials like whey lactose, coals and methane. Steinbuchel in 2001 mentioned that in order to produce PHAs other than PHB from CO_2 or renewable resources it will be necessary to link central metabolic pathway with PHA synthesis [15].

Rehm et al., determined that the gene product of *phaG*, (*R*)-3-hydroxyacyl-ACP:CoA transacylase, is responsible for the conversion of (*R*)-3-hydroxyacyl-ACP to (*R*)-3-hydroxyacyl-CoA [140]. Another example, the citric acid cycle for generating PHA precursors in recombinant *E. coli*; a polymer consisting of 4-hydroxybutyrate (4HB) was produced by drawing succinyl-CoA from the citric acid cycle, which is converted to 4-hydroxybutyryl-CoA, a precursor for PHA biosynthesis [141] as illustrated in Fig. 5.

8.3.3.2 Examples of Metabolic Engineering

Wild type *P. putida* is able to produce PHA_{MCL} from related and unrelated substrates [related = eg. fatty acid, unrelated = eg., glucose or gluconate] [46, 130, 131, 139]. An isolated *P. putida* mutant can grow on any carbon source tested and was able to synthesize PHA_{MCL} from fatty acids but not from gluconate or glucose [140]. Biochemical studies on the affected enzyme revealed that these enzyme catalyses the transfer of (*R*)-3-hydroxydecanoyl moieties from the acyl carrier protein: coenzyme A transferase. The respective gene, which complemented the mutant, was referred to as *phaG* [140], this one has been also identified and cloned from *P. aeruginosa* [142] and *P. oleovorans* [143] (Fig. 5).

In the presence of the substrate, (*R*)-3-hydroxyacyl-CoA the transacylase appeared in apparent molecular weights of 45.4, 83.7 and 102 kDa, which indicated substrate-induced conformational changes and oligomerization of the transacylase [144].

8.3.3.3 Metabolic Engineering using *E. coli* fadB Mutants

In *E. coli* fadB mutant LS1298, the dehydrogenase function is defective, whereas the hydratase and also the epimerase function of FadB remain active [145]. When the fadB mutant LS1298 of *E. coli* harboring *phaC1* was used for PHA biosynthesis, PHAs were accumulated to a significant level, contributing to 20-25% of the cell dry matter [146]. Obviously, (*S*)-3-hydroxyacyl-CoA accumulates in the cytoplasm of the mutant cells to a level that is sufficiently high enough to be effectively converted into the (*R*)-stereoisomer

and subsequently incorporated into PHA. Instead of *phaC1*, *phaC2* of *P. aeruginosa* was also used, and very similar results were obtained [147].

The length of the incorporated 3-hydroxyalkanoic acids reflected the length of the carbon chain of the fatty acid provided as carbon source [147]. The $fadBA_{PO}$ operon and a class II PHA synthase gene of *P. aeruginosa* were heterologously co-expressed in *E. coli*. FadBA$_{PO}$ did not mediate PHA$_{MCL}$ biosynthesis in *E. coli* wild type strain harboring a PHA synthase gene when cultivated on fatty acids as carbon source. However, PHA accumulation was strongly impaired in a recombinant *E. coli* fadB mutant, which harbored a PHA synthase gene. These data indicates that pseudomonads FadBA does not possess the inherent property, based on a putative epimerase function, to provide the (*R*)-enantiomer of 3-hydroxyacyl-CoA efficiently and that other linking enzymes are required to efficiently channel intermediates of *β*-oxidation towards PHA$_{MCL}$ biosynthesis [148].

8.3.3.4 (*R*)-Specific Enoyl CoA Hydratase

In *A. punctata*, a (*R*)-specific enoyl-CoA hydratase is responsible for the conversion of trans-2-enoyl-CoA to (*R*)-3-hydroxyacyl-CoA during the cultivation of this bacterium on e.g. hexanoate [149]. Two (*R*)-specific enoyl-CoA hydratase genes were also identified in *P. aeruginosa* [150]. Studies in recombinant strains of *E. coli* provided evidence that fabG of fatty acid *de novo* synthesis, which is a 3-ketoacyl-ACP reductase, is non-specific and exhibits activity with the corresponding CoA-thioesters [151]. On the other hand, it was shown that recombinant strains of *E. coli* could also use their own *β*-ketothiolase (FadA) in combination with the acetoacetyl-CoA reductase (PhaB) of *C. necator* for the conversion of fatty acids into PHA$_{MCL}$ [152] Figure 5.

8.3.3.5 Metabolic Engineering using Inhibitors

Steinbüchel described six chemicals, which specifically inhibit one or other reactions that affect PHAs production [15]. Whereas acrylic acid [153] inhibits the *β*-ketothiolase of the *β*-oxidation pathway, thiolactomycin [154], 3-decenoyl-N-acetylcysteamine [155], triclosan [156] diazaborine [157], and cerulenin [158] inhibit enzymes of the fatty acid *de novo* synthesis pathway.

A different system for the production of PHA$_{MCL}$ by *E. coli* was described using acrylic acid as inhibitor [159]. When recombinant strains of *E. coli*, expressing either *phaC1* or *phaC2* from *P. aeruginosa* were cultivated on fatty acids such as decanoic acid and in the presence of low concentrations of acrylic acid, PHAs were found to be accumulated in all investigated strains of *E. coli* [159].

Acrylic acid and cerulenin were applied to investigate the competition of the pathways for PHB-*co*-PHV and triacylglycerol (TAG) biosynthesis in species of the genus *Rhodococcus* [160]. Acrylic acid completely inhibited the accumulation of TAGs from valerate and caused a two-fold increase in the PHA content which consisted exclusively of 3HV [160].

Antonio et al., and Amara et al., produced 3HB-3HHX from cultivation of recombinant *E. coli* on fatty acid using PhaC class I and acrylic acid [101, 161].

9. *IN VITRO* BIOSYNTHESIS OF PHAs

Gerngross and Martin have first demonstrated *in vitro* biosynthesis of PHA and self-assembly of spherical granules by only using purified polyester synthase and substrate [162]. Poly(3HB) was also synthesized by employing the PHA synthase of *A. vinosum* in combination with the auxiliary enzymes propionyl-CoA transferase from *Clostridium propionicum* alone or with an acetyl-CoA synthase of *Saccharomyces cerevisiae* [163].

In vitro PHA biosynthesis was achieved using purified PHA synthases alone or together with additional auxiliary enzymes purified from various sources. The auxiliary enzymes over purified ones help to use substrates other than coenzyme A thioesters of hydroxyalkanoic acids and to recycle coenzyme A, which must then be used only in catalytic instead of stoichiometric amounts.

PHB was synthesized from 3-hydroxybutyryl-CoA using the PHA synthases from *C. necator* [162, 164] or *A. vinosum* [165]. PHV was synthesized from 3-hydroxyvaleryl-CoA using the PHA synthase of *C. necator* [166].

Homopolyesters and copolyesters of various SCL poly(HA_{SCL}) such as 3HB, 4HB; and 4-hydroxyvalerate (4HV) were synthesized by employing the PHA synthase of *A. vinosum* plus the butyrate kinase and the phosphotransbutyrylase of *Clostridium acetobutylicum* as auxiliary enzyme [167].

PHD was synthesized by using the PHA synthase PhaC1 of *P. aeruginosa* plus an acyl-CoA synthase of *Pseudomonas sp.* [168].

10. PHA PRODUCTION IN YEAST

Reush, 1989 describes an evidence of PHB existing in yeast and many other eukaryotic cells, which contain small amount of low molecular mass PHB, which function as complexes with polyphosphate in membrane transport [169].

Expression of the *C. necator* PHB biosynthetic pathway was successfully achieved in the yeast *Saccharomyces cerevisiae* by Leaf et al.,who reported that the expression of PHB synthase in the yeast cytoplasm is sufficient for PHB accumulation and that wild type yeast synthesis D-3HB-CoA could rise from intermediates in fatty acid synthesis or through β-oxidation [170]. Yeast cells can be used as models to gain information about PHAs synthesis in eukaryotes [171]. It is confirmed that three thiolase exist in *S. cerevisiae* functioning in mitochondria, cytoplasm [172] and peroxisomes [173]. Safake et al, in their study succeeded to isolate PHB producing wild type yeast strain from Kombucha tea and reported that the amount of PHB is less than that from prokaryotic and the PHB amount depend upon the type of culture and species [174].

Synthesis of PHA_{SCL} was established in the peroxisome of a wild-type yeast strain by targeting the *C. necator*$_{SCL}$ polymerase to the peroxisome [175].

11. PHAs PRODUCTION BY TRANSGENIC PLANTS

Plants are interesting targets for production of the biopolyesters. Transgenic plants could produce PHAs directly from CO_2 and solar energy [176]. The biosynthesis of PHB

in transgenic *Arabidopsis thaliana* was already reported [3]. Later, the *C. necator* PHB biosynthesis genes were also expressed in agricultural crops such as *Brassica napus* [177], *Gossypium hirsutum* [178] and *Nicotiana tabacum* [179]. The copolyester PHB-*co*-PHV [180] and PHAs consisting of $3HA_{MCL}$ [181] have been produced in plants.

12. ORGANIZATION OF PHA SYNTHASE GENES

The PHA biosynthesis genes and genes for other proteins related to the metabolism of PHA remain in cluster in bacterial genomes. In *C. necator*, the genes for PHA synthase (*phaC*), *β*-ketothiolase (*phaA*) and the NADP-dependent acetoacetyl-CoA dehydrogenase (*phaB*) constitute the *phaCAB* operon [132, 134, 138]. Approximately 4 kbp downstream of this operon a second *β*-ketothiolase gene (*bktB*) was identified [138]. In *Zoogloea ramigera*, *Methylobacterium extorquens*, *Sinorhizobium meliloti* 41, *Nocardia corallina*, *R. ruber*, *Paracoccus denitrificans*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum* Ha, *Rhodobacter capsulatus* and *Aeromonas punctata phaC* seem to be separated from other *pha* genes.

phaA, *phaB* or other genes related to PHA metabolism are not directly linked to the *phaCs* genes in these genomes. In contrast, the gene encoding an enoyl-CoA hydratase in *A. punctata* is located downstream of *phaC*. *P. oleovorans*, *Pseudomonas sp*. 61-3 and *P. aeruginosa* posses two different *phaC* genes, which are in the genome separated by the structural gene for an intracellular PHA, depolymerase. *Pseudomonas sp.* 61-3 contains also these two *phaC* genes in addition to another *phaC* gene, which is co-localized with *phaB* and *phaA*.

In all bacteria, which posses a two-component PHA synthase, *phaC* and *phaE* are directly linked in the genomes constituting most probably single operons. In *Allochromatium vinosum phaA* and *phaB* are located on the opposite direction in a gene cluster related to PHA metabolism. The organization of the genes is most probably similar if not identical in *Thiocystis violacea* and *Thiocapsa pfennigii*, whereas in *Synechocystis sp*. PCC 6803 further *pha* genes do not map close to the *phaEC* locus. In *R. ruber* and *A. vinosum* the structural genes for phasin proteins (ORF3 and ORF5, respectively) map close to the respective *phaC* loci [182].

13. PRIMARY STRUCTURES OF PHA SYNTHASES, CLASSES AND SUBSTRATE SPECIFICITY

PHA synthases are classified into four classes based on their substrate specificity and subunits composition. The class I PHA synthases, with *C. necator* synthase as prototype, is composed of one single type of polypeptide chain and use mainly (*R*)-3-hydroxybutyryl-CoA, (*R*)-3-hydroxyvaleryl-CoA and other SCL hydroxyalkanoic acid CoA thioesters including 3-mercaptoalkanoic acid CoA thioesters as substrates [16, 182]. The class III PHA synthases, as represented by the *A. vinosum* enzyme, is composed of two different subunits each of about 40 kDa [183]. The substrate specificity is similar to that of class I synthases, although some medium chain length 3-hydroxyfatty acids are also incorporated [184].

Both classes (I and II) of PHA synthases were purified, and *in vitro* activity has already been achieved [162, 163, 185, 186]. The class II enzymes, which are composed of only one type of subunit, are mainly found in pseudomonads such as *P. aeruginosa*. One major difference between class II and both, class I and III PHA synthases is the substrate specificity. Class II PHA synthases incorporate preferentially 3-hydroxyfatty acids of MCL (C6-C14) into PHAs, and the resulting product is a latex-like polymer [147, 159]. These substrates are mainly derived from intermediates of fatty acid *β*-oxidation or from fatty acid *de novo* biosynthesis [140 - 143, 188] provided fatty acids or simple non-related carbon sources [141]. Class II PHA synthases were purified and *in vitro* activity was achieved [189, 190]. An amino acid sequence alignment showed that the *A. vinosum* PHA synthase revealed high homology to prokaryotic lipases whose crystal structures are known. The amino acid Ser at the active site of the lipase did align with the Cys at the active site of the PHA synthase. A threading model of the *A. vinosum* PHA synthase was generated [191]. Accordingly, a threading model of the class II PHA synthase from *P. aeruginosa* was developed [192] and based on the homology to the structure of the lipase from *B. cepacia*. Rehm *et al*. developed threading model for PHA synthase from *C. necator* based on the alignment of the *C. necator* PHA synthase ($PhaC_{Re}$) with the *Burkholderia glumae* lipase (1TAH_A) [193].

In the multiple alignment of PHA synthases only one Cys residue (Cys-319) is highly conserved. Therefore, scientists are looking since several years for the second thiol group. The essential role of Cys-319 of the *C. necator* PHA synthase for the reaction mechanism was obtained from site-specific mutagenesis [185]. The weakly conserved Cys-459 was supposed to be involved in the catalytic cycle, providing the second thiol group. However, site-specific mutagenesis [185] clearly indicated, that this amino acid residue is not essential for catalytic activity. This is fully supported by the alignment of PHA synthase sequences. In position 260 of the PHA synthase from *C. necator* a conserved Ser residue was observed. This Ser-260 was proposed as target for covalent posttranslational modification by 4-phosphopantetheine, which should provide the second thiol group similar to fatty acid synthase. In order to investigate posttranslational modification by 4-phosphopantetheine, radiolabeling experiments were conducted, expressing PHA synthase gene from *C. necator* in *E. coli* SJ16 (*panD*). Since *E. coli* SJ16 is *β*-Ala auxotroph, specific radiolabeling of 4-phosphopantetheinylated proteins occurs, when cells were fed with 2-14C-*β*-Ala. These experiments indicated that the PHA synthase was labeled by 4-phosphopantetheine [185].

However, detailed analysis revealed that only a small portion of total PHA synthase was labeled [194]. Functional low level expression of PHA synthases from *C. necator* and *P. aeruginosa*, respectively, in *E. coli* SJ16 and also in *β*-alanine auxotrophic mutants of *C. necator* with subsequent analysis of 4-phosphopantetheinylated proteins gave no evidence for covalent posttranslational modification by 4-phosphopantetheine [195]. Exchange of amino acid residue Ser-260 against alanine and threonine, respectively, abolished *in vivo* and *in vitro* activity of PHA synthase from *C. necator* [195]. The current model of active PHA synthase involves two subunits forming a homodimer PHA synthases have been assigned to four classes based on their substrate specificity and subunit composition. The multiple alignments of the primary structures of these PHA synthases showed an overall identity of 9 to 96% with only 8 strictly conserved amino acid residues [193].

Considering class I and II PHA synthases, and forming a heterodimer (PhaC and PhaE) in case of class III PHA synthases. Accordingly, class I and II PHA synthases possess two thiol groups provided by conserved Cys-319, and in class III PHA synthases the second thiol group might be provided by conserved Cys-130 of subunit PhaE from *A. vinosum* [194].

A novel PHA synthase (class IV) from *Bacillus megaterium* required $PhaC_{Bm}$ and $PhaR_{Bm}$ for activity *in vivo* and *in vitro* was reported [196]. $PhaC_{Bm}$ showed greatest similarity to the PhaCs of class III in both size and sequence. Unlike those in class III, the 40 kDa PhaE was not required, and further more, the 22 kDa $PhaR_{Bm}$ had no homology to PhaE.

Amara and Rehm described seven site directed mutations have been performed in $PhaC_{Pa}$ where 5 conserved residues were replaced in order to identify the role of these amino acids in catalysis. The replacement of W398 by alanine abolished PHA_{MCL} synthase activity indicating that W398 is essential for enzyme activity [197].

The replacement of H479 by glutamine did not affect the PHA_{MCL} activity, which indicates that H479 is not the general base catalyst that activates the nucleophilic C296 for covalent catalysis. The replacement of C296 with serine which is the the general base catalyst in lipases did not abolish PHA_{MCL} synthase activity while still exhibiting 20% activity in comparison to the wild type. Replacement of C296 with Ala abolished PHA_{MCL} synthase activity which is a strong evidence that serine may be able to replace Cys as catalytic nucleophile for changing substrate specificity of PHA synthase. For further investigation, another site directed mutagenesis has been done in another conserved histidine (H452), which was replaced with Glu. The H452Q mutant was highly impaired in PHA_{MCL} activity, which indicates that H452 plays a major role as a general base catalyst instead of H479 [197].

Localized semi-random mutagenesis has been used to alter substrate specificity of the class II polyester synthase from *P. putida* [198]. Recently, Tsuge et al., found that the A510 of the *C. necator* polyester synthase is involved in substrate specificity [199].

The tertiary structure of PHA synthases has not been resolved by X-ray diffraction analysis, yet. Secondary structure prediction based on the multiple alignment of PHA synthases indicated with an expected accuracy of about 72%, that PHA synthases are mainly composed of variable loop (49.7%) and α-helical (39.9%) secondary structures, whereas for only 10.4% a β-sheet structures were predicted [200].

Thus, PHA synthases belong with respect to their predicted secondary structure content to the mixed class of proteins. Purified PHA synthase exists in an equilibrium of monomeric and dimeric forms whereas dimerization is significantly induced in the presence of substrate and of trimeric CoA analogs (3-hydroxybutyryl)3-CoA, respectively. In addition, the enzymatic lag phase is reduced and the specific activity increased in the presence of trimeric analogs [201]. This indicates that the dimeric form is substantially more active than the monomeric form in the absence of the putative primer. Since radiolabeled trimeric CoA analogs were found to be covalently bound to the PHA synthase

of *C. necator*, the radiolabel resides only the dimeric form as indicated by size-exclusion chromatography [202].

This homogenous population of particles measuring 11.2-12.8 nm in diameter and data derived from gel filtration chromatography indicate that this PHA synthase might be composed of ten subunits [186, 203].

Overall, with respect to quaternary structure of PHA synthases these data suggest that *in vitro* the active PHA synthase consists of two subunits and that *in vivo* the PHA synthase associated with PHA granule surface might be composed of ten subunits in *A. vinosum* [192].

14. PHA BIODEGRADABILITY

Biodegradation is a process mediated by special enzymes which able to degrade chemical compounds. Without enzymatic degradation, our life will not be imaginable. While depolymerases have been studied for about 40 years, the first structural gene of a PHA depolymerase (*Alcaliginus faecalis*, $phaZ_{Af}$) was cloned and sequenced only in 1989 by Saito et al. [204]. Depolymerases are highly specific for the polymers consisting of monomers in the (*R*) configuration [205]. PHAs depolymerase are carboxyesterases (EC 3.1.1) and hydrolyze the water insoluble polymer to water-soluble monomers and/or oligomers and finally to water and carbon dioxide or methane. Early PHB degrading bacteria were isolated by selection for microorganisms able to utilize PHB as the sole source of carbon and energy (Fig. 6) [24]. PHAs degrading bacteria differ from each other depending on the type of PHAs they degrade, however some bacteria revealed a rather broad polyester specificity and are able to utilize a wide range of PHAs [206, 207]. Three intracellular PHA depolymerases (PhaZ1, PhaZ2 and PhaZ3) and a 3HB-oligomer hydrolase (previously designated PhaZ2), which hydrolyse PHAs and the cleavage products produced by PhaZs, were cloned and characterized in *C. necator* [207 - 210].

Fungi also could degrade PHAs and their depolymerases have been characterized [211, 212]. PHAs depolymerases show a similar characteristic, they are stable at a range of pH, temperature, relatively small Mr (< 70 kDa), they are inhibited by reducing agent, e.g. dithiothreitol (DTT), which indicates the presence of essential disulfide bonds, and by serine hydrolase disulfide bonds, and serine hydrolase inhibitors such as diisopropyl fluorophosphate (DFP) or acylsulfonyl derivatives [212].

Depolymerases have three strictly conserved amino acids: serine, aspartate and histidine. The serine is part of the lipase-box pentapeptide Gly-Xaa1-Ser-Xaa2-Gly [213]. The oxygen atom of the serine side chain is the nucleophile that attacks the ester bond [206, 214].

14.1 Biotechnological Aspect of PHA Depolymerases

The production of enantiomer-pure (*R*)-hydroxyalkanoic acids by depolymerase-catalysed hydrolysis of PHA is possible.

The activities of PHAs depolymerase may vary depending on the composition and the physical form of the polymer (amorphous or crystalline), the environmental conditions and the dimensions of the sample [215 - 218].

While PHA can be produced by different fermentation processes based on agricultural feedstock's derived from CO_2 and water, their breakdown products by the activity of depolymerase are again CO_2 and water.

Thus, while in some applications the biodegradability is critical, PHAs are good renewable compounds [219].

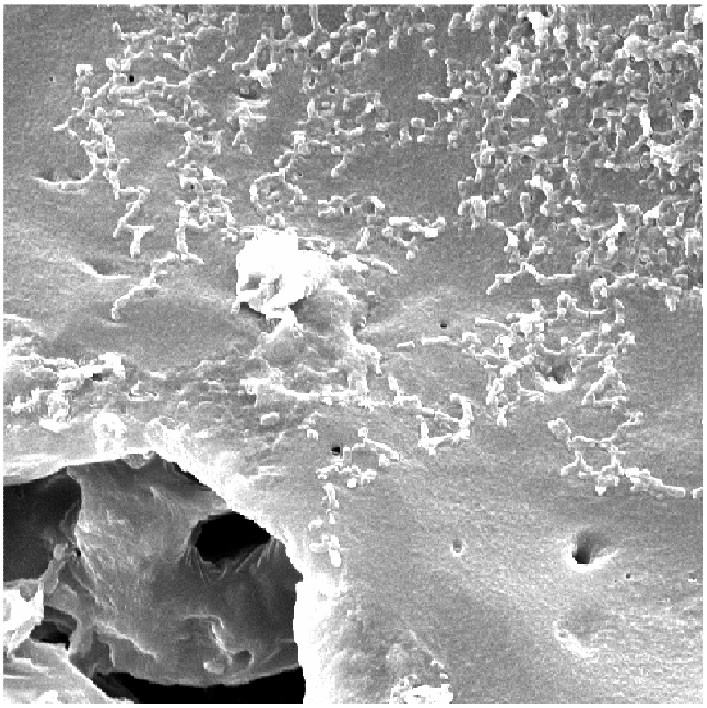

Fig. 6: Scanning Electron Microscope image for PHA surface [2000 x]. The bacterial growth leads to polymer degradation

15. PHA GRANULES

PHAs granules are composed of spherical shell-core particles of a PHAs core enclosed by phospholipids and proteins.

In vivo soluble PHAs synthases are changed into amphipathic enzymes upon the elongation of the water-insoluble PHAs chains, which remain covalently attached to the enzyme. A self-assembly process suggested to be initiated and leads to the formation of insoluble cytoplasmic inclusions with a phospholipid monolayer and covalently attached PHAs synthases at the surface. The structural proteins called phasins can be found attached to the granule surface [210, 220, 221].

Dunolp and Robards developed the first models for the structure of the inclusion with intriguing observation that this polymer was stretchable even at very low freeze -fracture temperature [222].

It was shown that PHA granules consists of a hydrophobic core of amorphous PHA that is surrounded by a membrane consisting of PHA synthases, the PHA depolymerase, amphiphilic phasin proteins and possible additional proteins. According to one model, these proteins are embedded in or associated with a phospholipid monolayer [221]. According to Byron, about 8 to 13 granules per cell having diameter range of 0.2 to 0.5 found in *C. necator* [223]. Immunoelectron microscopy studies of granules isolated from *A. vinosum*, employing gold-labeled anti-PhaC antibodies clearly indicated the presence of PHA synthase complexes at the surface of the PHA granule [189, 203].

Another model outlines a much more complex membrane structure with two phospholipids membranes [224]. NMR analysis provided evidence that water molecules are present in the core structure of the granules and that these molecules function as plasticizer [225]. Thus, the enzyme(s) responsible for PHA biosynthesis and consumption operate only on mobile hydrated material and that the solid granules characteristics of dried cells are partially artifactual.

Pötter et al. described orthologus and paralogous phasins occurring in *C. necator*. The analysis of the genome sequence of the PHAs accumulating bacterium *Ralstonia eutropha* (*C. necator*) strain H16 revealed three homologues (PhaP2, PhaP3 and PhaP4) of the phasin protein PhaP1. PhaP1 is known to constitute the major component of the layer at the surface of PHB granules. PhaP2, PhaP3 and PhaP4 exhibited 42, 49 and 45% identity or 61, 62 and 63% similarity to PhaP1, respectively [226].

In addition to PHA synthase, PHA depolymerases (PhaZ) are also bound to the PHA granule surface.

The establishment of *in vitro* polyester synthesis using purified polyester synthases form various microorganism, such as e.g. from *C. necator*, *A. vinosum*, *P. aeruginosa* (PhaC1 and PhaC2) and *P. oleovorans* (PhaC1) [163, 167, 168, 201, 227] provided a new tool to study the formation of macroscopic PHA granules. *In vitro* PHA synthesis was firstly achieved using recombinantly produced and purified *C. necator* polyester synthase [162]. The granule formation occurred after only a few minutes when the substrate, (*R*)-3-hydroxybutyryl coenzyme A, was provided to the purified polyester synthase [228].

16. PHA FUTURE

It is clear from the amounts of research performed on PHA indicates that this biopolymer is very promising. What made this kind of polymer has such attraction? It is the PHA properties. PHA is biodegradable, biocompatible and has good mechanical properties. PHA different technological aspects including production, purification, analysis, different applications, changing monomer composition etc., become clear based on the intensive research done concerning PHAs. Every week there is new information about PHAs. The number of publications exceeds most of the current important research topics.

What makes PHAs especially important is that the petroleum oil price is increasing tremendously and its stock will be ended approximately in this century! It is important for the global community to have a standby solution for the products derived from petroleum oil. PHAs at least will be a solution for most of the industries, which depends on material made from plastics. PHAs are environmentally and human-friendly which will make them more suitable than petroleum-based plastic for many applications. The price of PHAs' production decreases significantly, while at the same time the petroleum oil price is constantly increasing. The gap between the petroleum oil and PHAs prices becomes so close.

PHAs have promising futures in the field of biomaterial. The chemical, physical and biological properties of PHAs become very clear. The *in vivo* and *in vitro* production, controlling their monomer composition and the huge amount of their physical, chemical and biological properties increase the number of their applications.

Perhaps, it is suitable nowadays to use PHAs in cost effective products like medical instruments. Interesting progress in PHAs research has been recognized in the last ten years. From the literature, one can see that the developing countries have now an increasing interest in PHAs' researches. It might be useful to gain the benefit from collaborating research aiming to decrease the production cost of PHAs. The developed countries could help a lot for PHAs commercialization, especially those nations that have natural resources as well as the necessary work forces.

Finally, the researchers have made a lot of effort to improve this scientific field. Highlighting the importance of supporting researches on PHA will bring the wealth for all humanity which is one of the aims of this review. PHAs are safe products and green in all points.

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