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review

Modern Biotechnological Polymer Synthesis: A Review

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Summary

The industrial implementation of cost- and eco-efficient production of bio-based polymeric materials such as polyhydroxyalkanoates (PHAs) or polylactic acid (PLA) requires the comprehension of all process steps. The article at hand provides an insight into recent advances in allocation, pretreatment and utilization of raw materials available for biopolymer production in different areas of the world. Further, the high potential and risks of applying continuous process conduction in comparison with batch and fed-batch fermentation mode are elucidated. It is shown that the process design for continuous PHA production strongly depends on the kinetic characteristics for growth and product formation of the applied production strain. In addition, the triggering of the biopolymer properties by fine-tuning of the polyester composition during biosynthesis is demonstrated. Here, the impact of certain process parameters like the partial oxygen tension on the intracellular metabolic fluxes and the supplementation of cosubstrates on the polyester composition are discussed. In addition, such specialists among microbes are presented that possess the metabolic prerequisites to accumulate high-quality copolyesters merely from cheap unrelated carbon sources without the necessity for supplying expensive cosubstrates. In the field of downstream processing, sustainable methods for product isolation during biopolymer production that do not have a negative influence on the environment are presented.

Key words: biopolymers, downstream processing, fermentation strategy, polyhydroxyalkanoates, process design, raw materials, white biotechnology

General: The Exigency for Bio-Based and Biodegradable Plastics

In order to ensure the safe and efficient distribution of goods worldwide, there is a rapidly increasing need for polymeric compounds acting as packaging materials. Furthermore, polymers have a growing importance as niche products for special applications as in the medical field.

The contemporary utilization of constrained fossil resources for the production of polymers provokes current problems like the greenhouse effect and the global warming. This is caused by the fact that these materials are utilized only during a relatively short time span. After that, they are often incinerated, elevating the atmospheric CO_2 concentration. The main problem arising from incineration of plastics is the same as for energy recovery from fossil feedstocks: carbon that was fixed during millions of years and within this time was not part of the natural carbon cycle is converted to CO_2, which can accumulate in the atmosphere, contributing to the mentioned climatic effects. In addition, incineration of plastics often generates toxic compounds (*1*).

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Besides, more and more waste of highly resistant plastics that are not incinerated is piled up every year. Recycling systems demand a certain degree of purity and a high sorting accuracy. In addition, the collection costs are fairly high, and recycling has a negative impact on the quality of the materials, such as an increase in brittleness (*1*).

In addition to these ecological considerations, the price of crude oil is unpredictably fluctuating, not least due to miscellaneous developments in the global political situation. This constitutes a factor of immense uncertainty especially for the highly petrol-dependent polymer industry. In 2008, the price per barrel rocketed to a new all-time-high of 147 US$. The situation changed at the beginning of 2009 with a price of 30 to 40 US$ per barrel, which is in high contrast to the price of more than 80 US$, as was the case only a few months later in November 2009. Data for the remaining amounts of fossil oil in the earth's interior are changing quickly due to advanced methods for tracing and discharging of mineral oils. Nevertheless, one day fossil feedstock will finally be depleted. This causes an increasing awareness of the involved industrial branches for the necessity of promoting novel production techniques based on renewable resources. With this 'white biotechnology', sustainable production of polymers, fine chemicals, bulk chemicals and fuels is achieved by the action of living organisms or parts thereof such as enzymes.

Polyhydroxyalkanoates as Future-Oriented Alternatives

Polyhydroxyalkanoates (PHAs) are polyesters produced by numerous prokaryotic strains from renewable resources like carbohydrates, lipids, alcohols or organic acids classically under unfavourable growth conditions due to imbalanced nutrient supply. In general, PHA accumulation is favoured by adequate availability of a suitable carbon source and a limiting supply of nitrogen, phosphate or dissolved oxygen or certain microcomponents like sulphur, potassium, tin, iron or magnesium.

If PHA-rich cells are investigated using a light-optical microscope, the biopolymer is visible as refractive granules. These granules have a typical diameter of 0.2 to 0.7 μm and consist of 97.7 % PHA, 1.8 % protein and 0.5 % lipids. Native PHA granules can be stained with Sudan Black B and more specifically with Nile Blue A or Nile Red. Further studies show that the strongly fluorescent dye Nile Red can be used to directly monitor PHA inclusions in growing colonies (*2*,*3*). Proteins and lipids form a membrane coat surrounding the core region and stabilize the transition between the hydrophobic core region and its hydrophilic surroundings. Every granule consists of at least a thousand polymer chains forming right-handed 2_1-helices with a twofold screw axis and a fibre repeat of 0.596 nm, stabilized by hydrogen bonds and van der Waals intramolecular forces (*4*). Decades ago it was generally believed that the core of the inclusion bodies was almost crystalline, explaining the brittle character of PHB. X-ray diffraction analysis underlined this theory. But later it has been shown by means of ^{13}C NMR spectroscopy that the bulk of PHB and poly(3HB-*co*-3HV) in intact cells is not crystalline, but a mobile amorphous state (*5*).

For the producing microbial cells, PHAs mainly serve as reserve materials for carbon and energy. To a minor extent, they also influence certain enzymatic mechanisms in the cells. Under the conditions of limitation of external carbon, these reserve materials can be mobilized, thus providing the cell with an advantage for surviving during starvation periods. In addition, they play a major role as endogenous carbon and energy source for the formation of spores (*Bacillus* sp.) and cysts (*Azotobacter* sp.) (*6*). Table 1 compiles representatives of microbial genera known as PHA producers.

If items made of PHAs are composted after their utilization as bioplastics, they are completely degraded to water and CO_2 as the final products of their oxidative breakdown by the catabolic action of various microorganisms. Here it has to be emphasized that these final oxidation products are the basic materials for the photosynthetic regeneration of carbohydrates by green plants. This demonstrates that, in contrast to petrol-based plastics, PHAs are embedded into the natural closed cycle of carbon. The range of applications for PHAs is not limited to simple packaging materials, but encompasses commodity items, materials for agro-industrial purposes and for pharmaceutical and medical applications.

Chemically, PHAs are polyoxoesters of hydroxyalkanoic acids (HAs). Until the 1960s, only 3-hydroxybutyrate (3HB) was recognized as a PHA-building block. In 1974, microbes were isolated from sewage water that contained PHAs containing 3HB, 3-hydroxyvalerate (3HV) and, to a minor extent, building blocks with more than 5 carbon atoms (*7*). This number increased tremendously with the extraction of PHAs from marine sediments that contained at least 11 different PHA components (*8*). Until 1995, 91 components forming thioesters that can act as substrates for PHA synthases had been detected. This number has increased until today up to more than 150 constituents. It has to be emphasized that most of these constituents (also lactic acid) have only been proven *in vitro* to be possible PHA building blocks (*9*). Most of them are optically active (important exception is 4-hydroxybutyrate, 4HB) and, due to the stereospecifity of the biosynthetic enzymes, *R*-configured; reports of any *S*-configured HAs do not exist. These monomers can have saturated, unsaturated, straight or branched side-chains. In general, these side chains are mostly aliphatic, but when cultivated on unusual substrates, certain microbes can synthesize PHAs with pendant functionalities like aromatic, halogenic, pseudohalogenic or alkoxy groups (*6*).

Poly-β-malic acid (PMA) constitutes a special biopolymer that, according to its chemical composition, can also be classified as a natural PHA (structure shown in Fig. 1). In contrast to other PHAs, which are accumulated by bacteria and archaea, PMA is produced by several eukaryotic microorganisms like yeasts and moulds. Due to its pendant carboxy groups that can also be chemically linked to further substituents, the polymer is highly water soluble and easily biodegradable. Additionally, the material shows excellent bioresorbability. This makes PMA of special interest for application in the pharmaceutical field, especially for controlled drug delivery (*6*).

Table 1. PHA-accumulating microbial genera

Acidovorax	*Escherichia*	*Pedomicrobium*
Acinetobacter	(wilde type)[d]	*Photobacterium*
Actinobacillus	*Ferrobacillus*	*Penicillium*[e]
Actinomycetes	*Gamphospheria*	*Protomonas*
Aeromonas	*Gloeocapsa*[a]	*Physarum*[e]
Alcaligenes[a,b]	*Gloeothece*[a]	*Pseudomonas*[a,b]
Allochromatium	*Haemophilus*	*Ralstonia*[a,b]
Anabaena[b]	*Halobacterium*[a,c]	*Rhizobium*[a,b]
Aphanothece[a]	*Haloarcula*[a,b,c]	*Rhodobacter*
Aquaspirillum	*Haloferax*[a,b,c]	*Rhodococcus*[b]
Asticcaulus	*Halomonas*[a]	*Rhodopseudomonas*
Axobacter	*Haloquadratum*[c]	*Rhodospirillum*[b]
Azomonas	*Haloterrigena*[c]	*Rubrivivax*
Aureobasidium[e]	*Hydrogenophaga*[a,b]	*Saccharophagus*
Azohydromonas	*Hyphomicrobium*	*Shinorhizobium*
Azospirillum	*Klebsiella*	*Sphaerotilus*[a]
Azotobacter[a,b]	(recombinant)	*Spirillum*
Bacillus[a,b]	*Lamprocystis*	*Spirulina*[a]
Beggiatoa	*Lampropedia*	*Staphylococcus*
Beijerinckia[b]	*Leptothrix*	*Stella*
Beneckea	*Legionella*	*Streptomyces*
Brachymonas	*Methanomonas*	*Synechococcus*[a]
Bradyrhizobium	*Methylobacterium*[b]	*Syntrophomonas*
Burkholderia[a]	*Methylosinus*	*Thiobacillus*
Caryophanon	*Methylocystis*	*Thiocapse*
Caulobacter	*Methylomonas*	*Thiococcus*
Chloroflexus	*Methylovibrio*	Thiocystis
Chlorogloea[a]	*Micrococcus*	*Thiodictyon*
Chromatium	*Microcoleus*	*Thiopedia*
Chromobacterium	*Microcystis*	Thiosphaera
Clostridium	*Microlunatus*[b]	*Variovorax*[a,b]
Comamonas[a,b]	*Microvoleus*	*Vibrio*
Corynebacterium[b]	*Moraxella*	*Wautersia*[a,b]
Cupriavidus[a,b]	*Mycoplana*[a]	(today *Cupriavidus*)
Cyanobacterium[b]	*Nitrobacter*	*Xanthobacter*
Defluviicoccus[b]	*Nitrococcus*	*Zoogloea*[a]
Derxia[b]	*Nocardia*[a,b]	
Delftia[a,b]	*Nostoc*	
Ectothiorhodospira	*Oceanospirillum*	
Erwinia	*Oscillatoria*[a]	
Escherichia	*Paracoccus*	
(recombinant)[a]	*Paucispirillum*	

[a] detailed knowledge about growth and production kinetics available
[b] accumulation of copolyesters known
[c] archaea
[d] PHA found in cell membranes
[e] eukaryotic genera with poly-β-malic acid (PMA) production known

According to the number of carbon atoms of their building blocks, PHAs are divided into three different groups:

- short-chain-length (*scl*) PHAs: 3–5 carbon atoms
- medium-chain-length (*mcl*) PHAs: 6–14 carbon atoms
- long-chain-length (*lcl*) PHAs: more than 15 carbon atoms (until today, only *in vitro* production has been described; *lcl*-PHA building blocks have not been detected yet in naturally occurring PHAs!).

After utilization, the products can either be recycled, composted or they can be hydrolyzed to high-price enantiomerically pure monomers acting as starting fine chemicals for organic synthesis of chiral compounds such as vitamins, antibiotics, aromatics, perfumes or pheromones. These chiral follow-up compounds typically possess higher market values than the polymers themselves (*10*).

The type of polyester to be produced is very much dependent on the strain. *Cupriavidus necator* (former *Wautersia eutropha, Ralstonia eutropha, Alcaligenes eutrophus*) or *Azohydromonas lata* (former *Alcaligenes latus*), for example, can polymerize only 3-hydroxyalkanoates (3HAs) consisting of 3–5 carbon atoms (producers of *scl*-PHAs). *Pseudomonas putida*, a representative of the so-called pink or fluorescent pseudomonades, only accepts 3HAs of 6–14 carbon atoms. This different behaviour is caused by the specificity of the PHA synthase, the enzyme responsible for polymerization of hydroxyalkanoic acid thioesters. Due to this high specificity of PHA synthases, it was believed for a long time that one strain can either produce *scl*-PHAs or *mcl*-PHAs. During the last years, some exceptions have been reported, *e.g.* the production of a poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) copolyester by strains of *Aeromonas* sp., when they were grown on fatty acids of more than 12 carbon atoms (*11,12*), or the production of a random copolyester of different 3HAs consisting of 4–12 carbon atoms from gluconate by a *Pseudomonas* strain (*13*).

A lot of work has been done during the last couple of years by a German group investigating polythioesters (PTEs) like poly(3-mercaptopropionate). These materials are biotechnologically accessible by recombinant prokaryotes starting from mercaptoalkanoic acids like 3-mercaptopropionate (3MP) as substrates for the fermentation process. Comparison of PTEs to the corresponding oxo--analogues (PHAs) revealed lower crystallinity of PTEs and in some cases higher thermal stability; these features make them interesting for different applications. It has to be emphasized that the preparation of the mercaptoalkanoic acids required as substrates is highly costly. Hence, an economical feasible production of these compounds on a larger scale within the next few years appears rather doubtful. In addition, no biodegradation of PTEs has been observed until today, only copolyesters of 3HB and mercaptoalkanoates like poly(3HB-*co*-3MP) are degradable by natural depolymerases due to their specifity for oxoesters. Hence, at the moment the positive impact of a broad implication of these materials on the ecological development still has to be questioned (*14*).

Fig. 1 shows the general structure of PHAs, a *mcl*--PHA harbouring functional groups, the chemically related polymers PLA and PMA and the general structure of PTEs.

Table 2 gives an overview of the occurrence and production of PHA, PLA, PMA and PTE and their corresponding monomers.

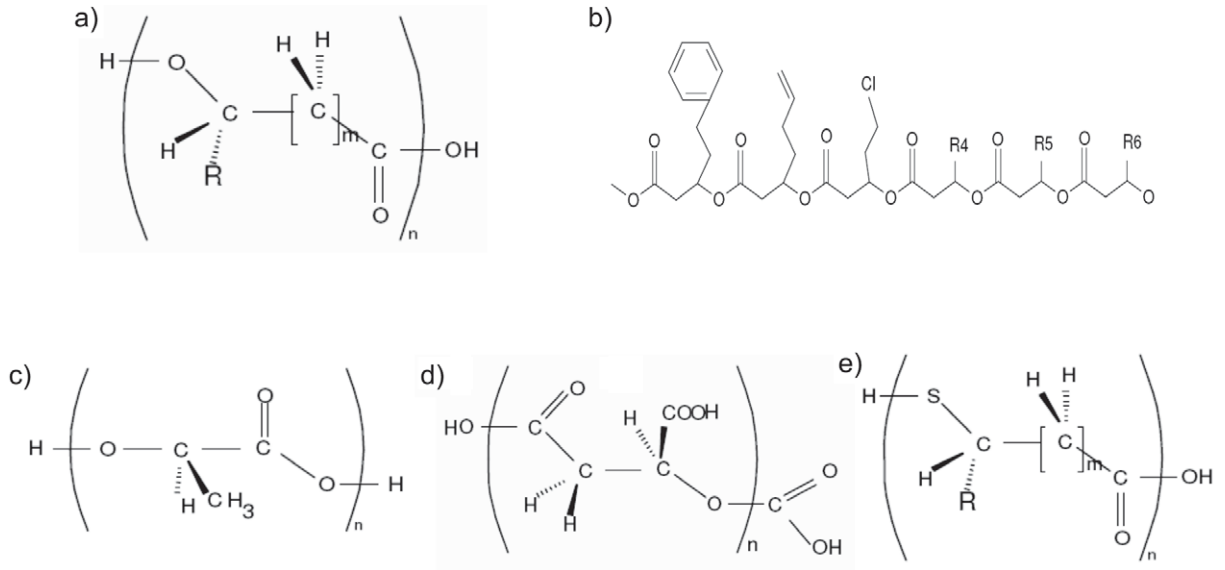

Fig. 1. Chemical structures of bio-based polyesters: a) general structure of PHA; b) example of a functional PHA harbouring 5-phenyl-pentane, 6-hexene and 5-chloro-pentane groups (R4 to R6 indicate additional functional PHA building blocks); c) poly-L-lactic acid; d) poly-β-malic acid; e) general structure of polythioesters (PTE)

Table 2. Characteristics of bio-based polyesters

Polymer	Monomer	Monomer production	Polymer production	Natural polymer degradation by depolymerases
PHA	hydroxyalkanoates	prokaryotes (archaea, bacteria), eukaryotes	prokaryotes (archaea, bacteria), eukaryotes*	+
PLA	lactate	prokarytes, eukaryotes**	PLA: only chemical, but production of poly(3-hydroxybutyrate-*co*-lactate) in genetically engineered strains	+***
PMA	β-malate	eukaryotes	eukaryotes	+
PTE	mercaptoalkanoates	chemical	prokaryotes	–****

PHA – polyhydroxyalkanoates
PLA – polylactic acid
PMA – poly-β-malic acid
PTE – polymercaptoalkanoates
*oligomers of 3-hydroxybutyrate also occurring in human blood and tissue
**anaerobic lactic acid formation in higher organisms
***total degradation of highly crystalline PLA doubtful
****only copolyesters of mercaptoalkanoates and hydroxyalkanoates degraded by natural depolymerases

The major advantages of PHAs can be summarized as follows:

- Biodegradability: It is a typical feature of biologically synthesized polymeric compounds to be again degraded by the action of living organisms. PHAs do not contribute to an increase of the landfill crisis due to their biodegradability; in contrast to conventional plastics, they can be composted after use.
- Biobased nature and independence from fossil fuels: Because PHAs are produced from renewable resources, they are independent from the availability of fossil feedstocks; if the generation of energy for the PHA production process itself is also based on renewable resources, the independence from fossil fuels is valid for the entire production process.
- Carbon balance: If PHAs are applied instead of fossil oil-originated polymers, the carbon cycle is closed. This is due to the fact that the carbon sources used for the biotechnological production derive from carbon dioxide that was previously fixed by green plants. Hence, the release of CO_2 by degradation of polyhydroxyalkanoates is just the final step of natural mineralization. The negative effects of CO_2 accumulation in the atmosphere, *e.g.* the greenhouse effect, will be related only to the amount originated from energy production and not to the carbon in the biopolymer itself.
- Biocompatibility: In special fields of applications (especially medical purposes like preparation of implants or surgical devices), PHAs are superior to

conventional plastics due to their biocompatibility. This is underlined by the natural occurrence of 3HB and its low molecular mass oligo- and polymers in human blood and tissue (*15–18*).

Fig. 2 shows electron microscopic pictures of *Cupriavidus necator* DSM 545 cells harbouring PHA granules obtained in a continuous fermentation of glucose.

Impact of the Biotechnological Production Strategy on the Polymer Properties

Effect of PHA composition on the material properties

Different applications require different material properties of the biopolyester. These properties can be triggered by fine-tuning of the composition of the PHA during biosynthesis. The most common representative of PHAs, namely the homopolyester PHB, features a high degree of crystallinity and restricted processibility of this material. The difference between the decomposition temperature (typically around 270 °C) and the high melting point (typically around 180 °C) provides quite a small window of processibility for melt extrusion technology. This can be changed by interrupting the PHB matrix through incorporation of alternative building blocks like 3HV or the achiral building blocks 4HB and 5-hydroxyvalerate (5HV). Such short-chain-length PHAs (*scl*-PHAs) feature the characteristics of thermoplasts like polypropylene. This is especially valid for PHB and its copolyesters with low amounts of 3HV, 4HB or 5HV. Table 3 (*19,20*) provides the material characteristics of some differently composed microbial PHAs, whereas Table 4 (*21–24*) indicates that there are many similarities, but also important differences in the properties of PHB and poly(3HB-*co*-3HV) on the one hand, and the petrochemical product polypropylene (PP) on the other hand. From the data presented in Table 4, it is visible that pure PHB possesses several disadvantages compared to PP. PHB is a stiffer and more brittle material (higher crystallinity) that has a lower resistance to solvents and a very low extension to break. The melting points and tensile strengths of PHB and PP are very similar, density of PHB is higher compared to PP. PP is not competitive with PHB concerning UV resistance, exclusion of oxygen and, most of all, biodegradability. This makes the material of special interest for application *e.g.* in the sector of food packaging, where bioplastic waste spoiled with food residues can easily be composted after use.

PHAs containing HAs with a higher number of carbon atoms than 3HV, so called medium-chain-length PHAs (*mcl*-PHAs), constitute elastomers with lower melting points, low glass transition temperatures and low crystallinity in comparison with *scl*-PHAs. Depending on the exact composition, *mcl*-PHAs often have the consistency and texture of resins or latex and do not really resemble the plastic-like *scl*-PHAs. In the case of functional *mcl*-PHAs harbouring unsaturated or other highly reactive groups, the possibility of post-synthetic modification *via* enzymatic and chemical methods is provided. This opens up new avenues in the field of tailor-made, high-performance biomaterials.

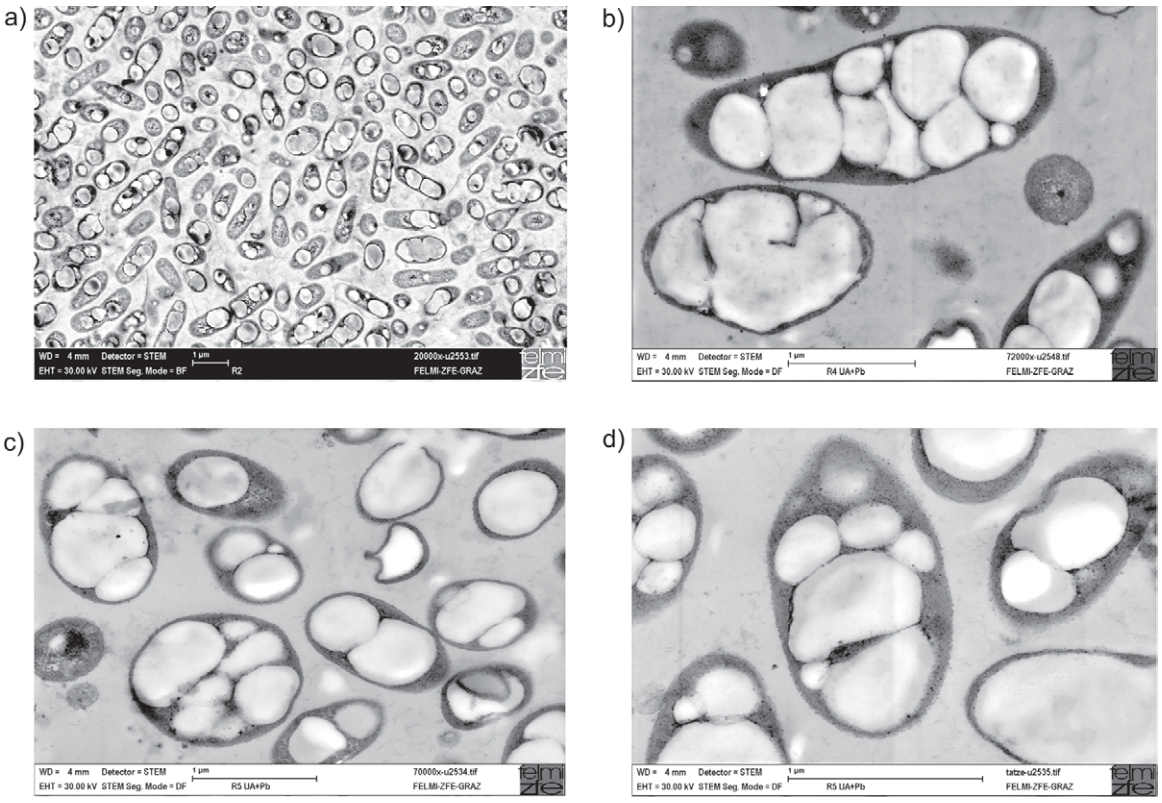

Fig. 2. Electron microscopic pictures of PHA-rich *Cupriavidus necator* DSM 545 cells cultivated in a continuous fermentation of glucose. Magnification: a) 1/20 000, b) 1/72 000, c) 1/70 000 and d) 1/150 000. Percentages of PHA in cell mass: a) 48 %, b) 65 % and c and d) 69 %. The pictures were kindly provided by Dr Elisabeth Ingolić, FELMI-ZFE, Graz, Austria

Table 3. Characteristics of representative PHAs (*19,20*)

	PHB	Poly (3HB-*co*-3 % 3HV)	Poly (3HB-*co*-20 % 3HV)	Poly (4HB)	Poly (3HB-*co*-3 % 4HB	Poly (3HB-*co*-16 % 4HB)	Poly (3HB-*co*-64 % 4HB	Poly (3HO-*co*-12 % 3HH)
melting temperature/°C	177	170	145	60	166	152	50	61
glass transition temperature/°C	4	–	–1	–50	–	–8	–	–35
tensile strength/MPa	40	38	32	104	28	26	17	9
Young´s modulus/GPa	3.5	2.9	1.2	149	–	n.d.	30	0.008
Elongation at break/%	6	–	50	1000	45	444	591	380

PHB – poly-3-hydroxybutyrate
3HB – 3-hydroxybutyrate
3HV – 3-hydroxyvalerate
4HB – 4-hydroxybutyrate
3HH – 3-hydroxyhexanoate
3HO – 3-hydroxyoctanoate
n.d. – not determined

Table 4. Comparison of the selected properties of PHB, poly(3HB-*co*-3HV) and polypropylene (*21–24*)

Property	PHB	Poly(3HB-*co*-3HV)*	Polypropylene
$M/(10^5$ g/mol)	1–8	3	2.2–7
$\rho/(kg/dm^3)$	1.25	1.20	0.905
melting point/°C	171–182	75–172	176
crystallinity/%	80	55–70	70
glass transition temperature/°C	5–10	–13–8	–10
O_2-permeability/$(cm^3/(m^2 \cdot kPa\ day))$	0.4	n.d.	17
UV-resistance	good	good	bad
resistance to solvents	bad	bad	good
tensile strength/MPa	40	25–30	38
elongation to break/%	6	8–1200	400
Young´s modulus	3.5	2.9 (3 % 3HV); 0.7 (25 % 3HV)	1.7
biodegradability	yes	yes	no

*for molar fraction of 3HV of 4–95 %
n.d. – not determined

The incorporation of different building blocks into the polyester chains normally requires expensive cosubstrates (precursors) and therefore constitutes a second decisive cost factor of major importance. These precursors do not only contribute to the production cost, but, in addition, are often toxic for the microbial strain. Therefore, the dosage has to be carefully controlled during cultivation (*25*). Here, a potential solution might be the utilization of such production strains that are able to produce special building blocks like 3HV from the unrelated carbon sources like simple sugars. Such organisms are found among osmophilic archaea like *Haloferax mediterranei* (*26*) and some species of *Rhodococcus*, *Nocardia* (*17,27,28*), some Pseudomonades (*25*), the activated sludge bacterium *Microlunatus phosphovorus* (*29*) and special non-sulphur cyanobacteria (*30*). Copolymers consisting of 3HB and 3-hydroxy-4-pentenoic acid (3H4PE) building blocks are reported to be produced by two strains of *Burkholderia cepacia* from the unrelated carbon sources like sucrose or gluconate (*31*).

Also in the case of *mcl*-PHAs, the formation of special building blocks from the unrelated carbon sources is described in literature. Sánchez *et al*. (*32*) report the formation of a PHA biopolyester consisting of different *mcl*-PHA components: 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxydodecanoic units from glucose or fructose as the sole carbon sources. Those building blocks with more than ten carbon atoms also feature non-terminal double bonds.

Impact of dissolved oxygen concentration on the incorporation of 3-hydroxyvalerate precursors

As mentioned earlier, precursor compounds for 3HV incorporation, namely odd-numbered fatty acids like propionic or pentanoic acid, increase the production price of the resulting PHA copolyester. The yield of 3HV units from propionic acid can be enhanced by restricted availability of dissolved oxygen (DO). This was demonstrated by Lefebvre *et al.* (*33*) using the strain *Cupriavidus necator*. Glucose and propionic acid were co-supplemented in the PHA production phase for biosynthesis of poly(3HB-*co*-3HV). In direct comparison with a control fermentation with sufficient oxygen supply (50–70 % of dissolved oxygen saturation during the accumulation phase), low DO experiments (DO from 1 to 4 % of air saturation) resulted in lower production rates for 3HB, probably due

to lower glucose uptake rates, while at the same time, the production rate for 3HV increased significantly, although the same enzymes were involved in 3HB and 3HV synthesis. Additionally, it turned out that higher yields of 3HV from propionate were achieved at low DO. The authors explained the described findings as follows: propionate is typically converted to propionyl-CoA, which further undergoes a condensation with acetyl-CoA (Ac-CoA) forming building blocks with five carbon atoms. By decarboxylation, propionyl-CoA can easily loose its carbonyl atom, thus building acetyl-CoA. Two Ac-CoA units are condensed forming 3HB. The experiments indicate that this unwanted oxidative loss of CO_2 from propionyl-CoA can be avoided under restricted oxygen supply, resulting in higher yields of 3HV from propionate at the expense of 3HB and, due to the lower glucose uptake, the total copolyester formation. On industrial scale, it has to be decided, as the case arises, if the increased 3HV yield from propionate, together with the minimized need for aeration, can economically compensate the lower overall PHA production. This will very much be dependent on the desired final composition of the copolyester (*33*).

Fine-tuning of the 4HB fraction in PHA

It was demonstrated that the conversion yield of the 4HB precursor γ-butyrolactone towards 4HB building blocks can be influenced by co-feeding of small amounts of precursors for 3HV production (propionate) or acetate. The 4HB molar fraction in the polyester could be increased from 38 to 54 % (*34*). The enzymatic background for these findings can be comprehended as follows: firstly, PHB synthase activity is induced by propionate; secondly, the Ac-CoA concentration also increases by supplying propionate and acetate. On the one hand, acetate is directly converted to Ac-CoA, while propionate, on the other hand, creates Ac-CoA by oxidative decarboxylation of propionyl-CoA. The increased concentration of Ac-CoA causes product inhibition on the lysis of 4HB-CoA to two molecules of Ac-CoA, thus providing more 4HB-CoA for the polymerization. It can be concluded that, by supplying propionate as a cosubstrate, the percentage of 4HB in poly(3HB-*co*-4HB) biosynthesis is determined by both a higher 4HB-CoA pool and an increased PHB synthase activity (*34*,*35*).

Polylactic Acid

Polylactic acid (PLA) constitutes another biobased polymeric material with properties of thermoplastics. The chemical structure of PLA is shown in Fig. 1. The materials are rather crystalline with a melting temperature of about 155 °C and are suitable for processing steps such as injection moulding, film-blowing and melt extrusion.

The commercial production of its monomer, lactic acid (2-hydroxypropionic acid) *via* chemical means is based on acidic hydrolysis of lactonitrile, which is classically generated by the conversion of acetaldehyde with hydrogen cyanide. In addition, the biotechnological production of lactic acid is also described in detail in literature (*36*). The desirable characteristic of an adequate lactic acid-producing strain is fast and complete substrate conversion with high yields of preferred stereospecific lactic acid under low pH value and high temperature conditions. Simply regarding the available raw materials, the choice of the strain primarily depends on the type of cheap carbon sources to be fermented. Here, numerous species of the genus *Lactobacillus* are known to convert sucrose, lactose, starch, or sulphite waste liquor (*36*,*37*). *Lactobacillus* is known to have special nutritional requirements due to its inability to synthesize its own growth factors. Therefore, the supplementation of complex additives such as dried blood is needed for an efficient generation of catalytically active cell mass for lactic acid production.

However, lactic acid cannot be directly polymerized to PLA, because each polymerization reaction generates one molecule of water, which degrades the formed polymer chain to low molecular masses. Instead, lactic acid is oligomerized and then catalytically dimerized to cyclic lactides. Although dimerization also generates water, it can be separated prior to polymerization. PLA of high molecular mass is produced from the lactide monomer by ring-opening polymerization (ROP) using chemical catalysts (*38*). This mechanism does not generate additional water, and hence, a wide range of molecular masses are available. For the polymerization reaction, high purity of the lactic acid is required. The optical purity of lactic acid is crucial to the physical properties of PLA, and an optically pure L(+)- or D(–)-lactic acid, rather than racemic DL-lactic acid, can be polymerized to a high crystalline PLA, which is suitable for commercial uses (*39*). In addition to the polymerization of lactic acid, the simple conversion to its methyl and ethyl esters results in another class of ecologically benign solvents.

Attempts have already been made to establish enzymes for *in vivo* polymerization of lactic acid as alternatives to the chemical catalysis. For this purpose, a lactic acid-CoA (LA-CoA) producing *Escherichia coli* strain has been developed *via* genetic engineering. This was accomplished using propionyl-CoA transferase, which transfers CoA from acetyl-CoA to lactate. After the production of LA-CoA by the strain had been confirmed, an engineered PHA synthase gene harbouring the information for β-ketothiolase and acetoacetyl-CoA reductase was inserted into the recombinant *Escherichia coli*. Using this strategy, it was possible to generate in a one-step biotechnological process a copolyester consisting of 6 % of lactic acid and 94 % of 3HB. In contrast, it was not possible to generate PLA homopolyester (*40*).

Depending on the envisaged field of application, it has to be decided, for each case separately, if the production of PHA or PLA is more reasonable. Due to the numerous possibilities of adjusting the composition, PHAs are more versatile in their properties than PLA, making them of interest for special applications as high-performance materials (*38*). The process of the production of PLA is more or less optimized; hence, they might be favourable for being utilized as 'simple' plastic materials in many fields. In any case, it has to be considered that PLA needs typically high temperatures of approx. 60 °C to initiate hydrolytic degradation; highly crystalline PLA is reported not to be biodegradable at all (*41*).

Feedstock as the Crucial Factor in Cost-Efficient PHA Biosynthesis

Types and occurrence of available feedstock

The application of biotechnological processes for industrial production can be regarded as promising for sustainable development, although for a range of products biotechnological production strategies have not yet passed the test of economic viability. This is often caused by the expenses of the raw materials. From the economic point of view, the fermentative part of the discontinuous production of 'simple' PHAs from purified substrates like glucose can be considered as optimized to a high extent. A viable strategy for cost-minimization can include the utilization of a broad range of waste and surplus materials that can be used as feedstock for the bio-mediated production of desired end-products. Such materials are produced mainly in agriculture or related industrial branches (*19*,*42*–*44*). The production of PHA is largely determined by the cost of raw materials due to the fact that PHA accumulation occurs under aerobic conditions, resulting in high losses of carbon substrate by intracellular respiration. Hence, not even 50 % of the carbon source is directed towards biomass and PHA formation. The utilization of waste materials for PHA biosynthesis is a good strategy for cost-efficient biopolymer production and helps industry to overcome their disposal problems (*45*).

The industrial scale conversion of value-added low-cost agricultural feedstock can provide a certain degree of geopolitical independence to many regions in the world. The selection of the appropriate waste stream as feedstock for biotechnological purposes mainly depends on the global region where a production plant will be constructed. In order to save costs of transportation, facilities for the production of biopolymers, biofuels and biochemicals should be integrated into the existing production lines, where the feedstock directly accrues as waste streams. The availability of convertible substrate has to be assured all year round. This brings up problems like the suitability for storage of these materials, especially for lignocellulosic materials during the off-season when no harvest takes place.

In Europe and North America, huge amounts of surplus whey are available in dairy industry, providing lactose for the production of PHAs, lactic acid, polylactic acid (PLA), bioethanol, and special chemicals like antibiotics or emulsifiers. Caused by new legislative situations, the increasing production of biodiesel in Europe generates enormous amounts of its major side stream, namely glycerol, a starting material for the production of PHA and lactic acid (*46*,*47*).

For the production of catalytically active biomass of particular microbial production strains, different waste streams show high potential as precious sources for nitrogen required for the formation of biomass constituents. Here, numerous protein hydrolysates, meat and bone meal from the slaughtering and rendering industries as well as several grass and silage residues show excellent results for the cultivation of microbes capable of the production of *e.g.* PHA (*45*,*48*).

In other areas of the world, waste from sugar industry, *e.g.* molasses (*49*), starch (*50*), waste lipids (*51*), alcohols like methanol (*52*) and especially lignocellulosic feedstock are available in quantities that are appropriate for industrial process demands.

The occurrence of waste lipids is versatile: waste cooking oil, different plant oils, meat and bone meal lipids or wastewater from the olive oil and palm oil production are available. In all cases, the triacylglycerides can either be directly utilized as a carbon source, or after hydrolyzation to glycerol and fatty acids, or after transesterification towards biodiesel and glycerol (*53*).

Lignocellulosic (consisting of lignin, cellulosic and hemicellulosic fibres) and cellulosic materials provide the largest amounts of feedstock. Industrial branches generating the major shares of this waste are wood processing, paper and agriculture industry. Nowadays, plenty of effort is dedicated worldwide to develop bio-refinery plants for the conversion of lignocellulose and cellulose waste to starting materials for biotechnological production of bioethanol, biopolymers and a range of fine chemicals. The optimization of methods for digestion of lignocellulose and the development of effective biocatalysts for the breakdown of cellulose and hemicellulose into microbially convertible sugars (hexoses and pentoses) are a prerequisite for an efficient biotechnological conversion of these promising raw materials into desired end products (*54*,*55*).

The integration of biopolymer production into an existing sugar cane mill is realized on pilot scale at the company PHBISA in Brazil, where the obtained saccharose is converted to bioethanol and partly to PHA. In this scenario, the required energy for bioethanol and biopolymer production is generated by burning of surplus biomass, namely bagasse. The fusel oil fraction (mainly iso-pentanol) from the bioethanol distillation is applied as extraction solvent for PHA isolation from microbial biomass (*56*).

Seasonal availability of the feedstock

In contrast to processes based on purified substrates, the utilization of waste materials for PHA production confronts industry with the question of availability of the feedstock during an entire production year. In general, distinction has to be made between such waste streams that accrue all over the year in more or less constant quantities, and others with an availability strongly fluctuating with time. The factor 'seasonal availability' is crucial for planning and designing of the PHA production facilities to be integrated into the existing industrial plants.

An example for the first case is found in the utilization of permanently accumulating surplus whey from dairy industry. Sizes of PHA production facilities to be integrated into the dairy process lines can easily be harmonized with the expected amounts of arising whey. Thus, the biotechnological conversion of whey to final products like PHA substitutes the disposal of this material in combination with value creation. Furthermore, classical processing steps of whey (*e.g.* production of dry whey or lactose) are energy demanding and show a low cost/performance ratio.

The situation changes fundamentally in the case of residues that accrue seasonally after the harvest and pro-

cessing of special agricultural crops. In this case, long periods without formation of the waste stream are interrupted by only one or a few time peaks a year, when large amounts of the material accrue. In such cases, the suitability of the raw material for low cost storage is of major importance. Otherwise large-scale PHA production plants would be needed to convert a huge amount of the waste material within a short time frame. Such large facilities are expensive and only operate at full capacity for very limited time periods. A solution to this problem is identified in the conversion of perishable materials into stable intermediates that can easily be stored without major energetic requirements like cooling, heating or drying. Lactic acid, the most widely occurring carboxylic acid in nature, constitutes a prime example of such stable intermediates.

Process Design

Classically, PHA production is accomplished under fed-batch feeding conditions, where substrates are supplied to the fermentation broth when required. Today, this addition can be automated to a high degree, *e.g.* by coupling of the substrate addition to process parameters like the dissolved oxygen tension, pH-value, the CO_2 concentration in the air leaving the bioreactor, or by automatic feeding under the precondition of a well-known kinetic situation of the process. But, due to its higher productivity, continuous production processes soaringly appear of special commercial interest, especially for fast growing microbial strains (*57–59*). This can be visualized by the following equation comparing the productivities for biomass in batch mode and continuous mode according to Eq. 1 (*60*):

$$\frac{Pr_{cstr}}{Pr_{dstr}} = \ln\frac{X_e}{X_i} + t_0\mu_{max} \qquad /1/$$

where Pr_{cstr} and Pr_{dstr} are the productivities of a continuous stirred tank reactor (CSTR) and a discontinuous stirred tank reactor (DSTR), respectively. X_e is the maximum biomass concentration, whereas X_i is the initial biomass concentration. t_0 specifies the so called 'dead time' of the production process needed for cleaning and refilling of the bioreactor, and μ_{max} is the maximum specific growth rate.

Using a continuous production system, t_0 can be set to 0 h instead of a minimum value of 10 h in a typical discontinuous process. This results in the possibility to substantially reduce the bioreactor volume in the continuous process for a desired fixed amount of product per unit of time. In discontinuous mode, μ_{max} is only obtained within a finite time frame during exponential growth, whereas in continuous mode, μ_{max} is obtained for the entire steady state of the process. For this steady state, the value of the specific growth rate corresponds to the value of the dilution rate D. From the engineering point of view, the reactor performance is also easier to control, because lower bioreactor volumes lead to less segregation by better mixing; additionally, lower expenditure for the energy supply is expected for lower reactor volumes (*61*).

To choose the adequate bioreactor design for continuous PHA production, kinetics for both biomass and PHA production by the microbial strain should be considered. In the case of PHA production directly associated with microbial growth as it is found in *Alcaligenes latus* DSM 1122 on sucrose (*57*), or in *Pseudomonas putida* ATTC 29147 on fatty acids (*62,63*), a one-step continuous process using a CSTR is a viable solution.

The situation changes significantly in the case of *Cupriavidus necator*, where autocatalytic growth of biomass is followed by a phase of linear PHA production; here, biomass production should occur in the first step in a CSTR, which is coupled to a plug flow reactor (PFR). The combination CSTR-PFR ensures not only higher productivity, but also minimizes the loss of substrates and cosubstrates. Furthermore, product quality can be enhanced by the fact that narrow residence time distribution is a characteristic of the PFR, leading to higher uniformity of cell populations. This should also have positive impact on the distribution of the PHA molecular masses and the composition of polyesters (*57*).

If the microbial strain (*e.g. Pseudomonas* sp. strain 2F on glucose) features hyper-production of PHA after a phase of carbon starvation, the combination of two subsequent CSTRs should be chosen as the optimum solution (*57*).

Besides the significance for industrial process development, continuous studies in chemostats are also a precious tool for elucidating the relationships between cells and their environment. For example, the optimization of the composition of nutritional media can be accomplished this way. In addition, continuous processes enable the fine-tuned supply of growth-inhibiting substrates.

On laboratory scale, continuous PHA production has been demonstrated also for *scl*-PHAs as for *mcl*-PHAs; single- and two-stage processes are reported.

The first studies on one-stage chemostat continuous PHA production were published by Ramsay *et al.* (*64*). *C. necator* was cultivated on glucose at a dilution rate of 0.15 h^{-1} and produced 5 g/L of biomass with a PHB content of 33 %. Similar results were obtained with *A. latus* using sucrose at the same dilution rate; biomass concentration and PHB content were 4 g/L and 40 %, respectively. When grown on glucose and various concentrations of propionic acid up to 5 g/L, *A. latus* produced poly(3HB-*co*-3HV) with the content of 3HV monomer in the PHA of up to 20 %. Using pentanoic acid instead of propanoic acid led to higher 3HV content in the copolymer. Assimilation of sucrose was inhibited when high concentrations of propionic acid were used (*64*).

Several other studies were conducted to produce the copolymer poly(3HB-*co*-3HV) with *C. necator* in one-stage continuous culture. Poly(3HB-*co*-3HV) was produced from fructose and propionic acid with a maximum PHA productivity of 0.31 g/(L·h), with a 3HV content in the range of 11 to 79 %. It was found that the molecular mass (M_n) increased with the dilution rate (*59*). When sodium propionate was employed as precursor for 3HV, the continuous culture systems did not reach significant steady states when the concentration of sodium propionate exceeded 7 g/L (*65*). Zinn *et al.* (*66*) grew *C. necator* in a

chemostat under the conditions of simultaneous limitations by carbon (butyric and/or valeric acid) and nitrogen (ammonium) source. No concentrations of biomass or polymer were reported, only the absorbance of the culture $A_{450\ nm}$=34±4 and the maximum 3HV content in the copolymer of 62 %. The study was rather focused on material properties, with the melting temperature of the material decreasing from 178 °C for PHB to about 80 °C for PHA with the highest HV content. The high relative molecular mass (M_r) of the obtained polymers was between 0.9 and $1.2 \cdot 10^6$ with a polydispersity index of around 3 (*66*).

In the case of *mcl*-PHAs, a Swiss group reports the continuous, growth-associated production of poly(3-hydroxyalkanoate-*co*-3-hydroxyalkenoates) in one-stage chemostat cultures of *Pseudomonas putida* ATTC 29147 in a single CSTR. The applied substrates encompassed 5-phenylvalerate, octanoate and 10-undecenoate. Multiple nutrient limited growth conditions were chosen at a dilution rate of D=0.1 h^{-1}. Different mixtures of the substrates in the feed resulted in the formation of copolyesters with varying compositions and different amounts of aromatic and unsaturated side chains that make the products accessible for further modification. The authors state that the steady state conditions in a continuous culture provide a strategy specially suited for the production of tailored PHA copolymers (*63*).

All single-stage experiments with *C. necator* resulted in rather low biomass concentrations and intracellular PHA contents, which is an expected outcome, since *C. necator* produces non-growth-associated PHAs. For such organisms, optimal conditions for both cell growth and PHA accumulation cannot be maintained in a single-stage system. For that reason, a multistage system should be more suitable, and in fact, the results from a two-stage chemostat set-up were superior to those from one-stage experiments. Using a system of two CSTRs, continuous PHB production with *Cupriavidus necator* was investigated by Yu *et al.* (*65*). Investigating the first stage, the best results were obtained at a dilution rate of D=0.21 h^{-1}, when 27 g/L of cell dry mass with about 11 % (by mass) of PHB were produced. As expected, the authors report high specific cell growth rates in the first stage and low PHB synthesis rates under carbon-limited and nitrogen-rich conditions. In the second stage, maximum PHB production was observed at a dilution rate of D=0.14 h^{-1}, giving a PHB concentration of 48 g/L. The maximal PHB productivity was reported to be 1.43 g/(L·h) at a dilution rate of 0.12 h^{-1}, but with relatively low PHB content of 47.6 % (*65*).

A few other studies in a two-stage continuous system were conducted with other microorganisms. In the case of poly(3HB-*co*-4HB) production, *Delftia acidovorans* P4a was cultivated on mixtures of acetic acid and γ-butyrolactone (GBL); here, poly(3HB-*co*-4HB) copolymers with a molar fraction of 2.7–19 % 4HB were obtained. The authors state that especially in the case of toxic substrates like acetic acid and GBL, the two-stage continuous production strategy is very convenient (*67*).

In the case of *mcl*-PHAs, *Pseudomonas oleovorans* was cultivated on octane with a volumetric PHA productivity of 1.06 g/(L·h) and PHA content of 63 % in the second fermentor. Dilution rates of D=0.21 h^{-1} in the first stage and 0.16 h^{-1} in the second stage were reported to result in the highest PHA productivity (*68*).

Similar to the production of poly(3HB-*co*-4HB) (*67*), continuous modes provide a viable strategy for the production of *mcl*-PHAs in order to overcome the high sensitivity of the production strains against higher concentration of the required substrates, mainly fatty acids and their derivatives. Here, continuous mode allows the permanent feeding of the organisms with such amounts of substrates to cover their metabolic requirements, but without reaching inhibiting concentrations.

Product Isolation

Downstream processing constitutes a key part of the entire PHA production process. After biosynthesis of the polyester and separation of the bacterial biomass (normally *via* centrifugation, sedimentation, flocculation or filtration), the needed process for PHA recovery constitutes another not negligible cost factor, especially in large scale production. Choosing the adequate method for separating PHAs from residual biomass is dependent on several factors: the production strain, the required product purity, the availability of isolation agents, and the acceptable impact on the molecular mass. Principally, three different strategies are described for PHA isolation:

- direct extraction of PHA from biomass (solvent-antisolvent methods; PHA is dissolved intermediary)
- chemical or enzymatic digestion of the non-PHA cellular material (PHA granules are set free, no intermediary dissolving of PHA)
- disruption of cells of osmophilic microbes in hypotonic medium (PHA granules are set free, no intermediary dissolving of PHA)

Direct extraction of PHA from biomass

Cost effectiveness of PHA isolation does not depend only on the equipment and chemicals needed, but, most of all, on the yields for product recovery and the possibility to reutilize the compounds needed for the isolation. For direct extraction of PHA from biomass, extraction solvents that can easily be recycled will be of interest (*69*). Solubility of PHAs, especially pure crystalline PHB, is a complex topic. Unlike most low molecular mass compounds and non-crystallisable amorphous polymers, the solubility of crystalline polymers such as PHB cannot be predicted from simple criteria, such as the similarity of chemical architecture, matching of refractive indices, dielectric constants or even solubility parameters, although such attempts are described in literature (*70*). For example, hexane is a non-solvent for linear PP, although both compounds are made of the same repeating units.

Typical halogenated extraction solvents like chloroform or, to a minor extent, dichloromethane or 1,2-dichloroethane at room temperature show excellent performance in isolation of short-chain-length *scl*- and *mcl*-PHA in terms of extraction yields and product purity (*71*). After the polyester is extracted from the biomass, its solubility is reduced by the addition of a PHA antisolvent, typically ethanol, methanol or acetone, resulting in the pre-

cipitation of highly pure PHA. Until some years ago, the remaining mixture of ethanol and chloroform had to be disposed of, because a reutilization of the solvents after separation *via* distillation was highly energy demanding. This is clearly in contrast to the aims of the biotechnological production of PHAs to be a sustainable, 'green' technology. Recently, a simple and effective method for separation of the mixture developed by the addition of water has been described. The three-component system water-chloroform-ethanol consists, at the right relative concentrations of the components, of two phases. The lower phase (95 % $CHCl_3$, residues of ethanol and water) can directly be reused for PHA extraction; the upper phase contains only negligible amounts of halogenated solvents (*72*).

The said halogenated solvents, especially chloroform, pose a high risk not only for the environment, but also for the personnel working with them. In order to avoid leaving the patterns of sustainability in biopolymer production, it will be indispensable to concentrate the development of new extraction processes on such recyclable solvents that are also of environmentally benign nature, such as lactic acid esters (*73*).

In Brazil, PHB is produced on pilot plant scale from sucrose, integrated into the multidimensional production line of a sugarcane mill, where not only PHB is produced from sugar cane sucrose, but also bioethanol. By-products of the bioethanol production are fusel alcohols like iso-pentanol, which is used as extraction solvent for PHB. After cell harvest, separation and concentration of cells results in a sludge containing 25–30 % of solids. This sludge then undergoes a multistage extraction process in continuous stirred tank reactors. Cell debris is removed and the remaining solution is cooled down, resulting in a gel. From this gel, the major part of the solvent is removed by mechanical and thermal treatment. The remaining solvent is removed by mixing the wet PHA with water and finally co-distilling the alcohols and the washing water. The extraction yield of the described process is 95 %. Although the purity of the material is reported to be higher than 98 %, the product possesses a yellowish to brownish colour and the remaining odour of the product. Additionally, a significant loss of molecular mass is observed (*56*).

Digestion of the non-PHA cellular material

An enzymatic digestion method has been developed by Imperial Chemical Industries (ICI; London, UK) to recover PHB from *C. necator* by proteases. This process includes thermal treatment of PHA-rich biomass, enzymatic disintegration of the cell material, and washing with an anionic surfactant to dissolve the residual biomass (*74*). Here, the costs for enzymes and the requirements of steps for increase of product purity are quite high.

Pseudomonas putida cells can be disrupted by a combination of heat shock and treatment of the cells with the enzyme alcalase, sodium dodecyl sulphate, and finally EDTA. By this procedure, the PHA granules remain unscathed (*75*).

Alternatively, the PHA-rich biomass can be, after a pretreatment with the surfactant Triton X-100, disrupted with sodium hypochlorite under strong alkaline conditions. This method turned out to be quite simple and effective, but resulted in an enormous reduction of molecular mass of the polyester of approx. 50 % (*76*,*77*).

When using recombinant *Escherichia coli* strains for PHA production, such cells become very fragile at high PHA content. This is due to the fact that *E. coli* is naturally not created for storing large amounts of reserve materials. This encouraged Choi and Lee (*71*) to develop a simple recovering method without freezing and drying step. A variety of surfactants, acids and hydroxides were tested, and the recovery yields and product purities were determined at different temperatures. Among the investigated chemicals, NaOH, KOH and SDS turned out to have sufficient recovery yields and product purities. In direct comparison, SDS resulted in the highest purities (97.9 % at 30 °C and 98.7 % at 37 °C), but the recovery yields were higher using the alkaline compounds. The authors also stated that the alkaline substances were cheaper and ecologically friendlier than SDS. The influence of the hydroxides on the molecular mass of PHB was nearly negligible (*50*).

A simple, cost-efficient and effective method for the recovery of PHB directly from high cell density culture broth without pretreatment steps has been developed by Kim *et al.* (*78*). This method consists of direct addition of SDS to the culture broth, shaking, heat treatment, and washing steps. When the SDS/biomass ratio was higher than 0.4, the purity of the recovered PHB was over 95 % at various cell concentrations. The recovery yield of PHB was over 90 % regardless of cell density and SDS concentration; the reduction in molecular mass was negligible (*78*).

In 1978, a patent was published by the Agroferm AG (Rothrist, Switzerland) claiming that cyclic carbonic esters such as ethylene carbonate and 1,2-propylene carbonate are particularly suitable solvents for PHB, especially for the extraction of PHA from moist protozoic and prokaryotic biomass. The biomass is suspended in the cyclic ester and heated under stirring. After removal of the hot extract, PHA is precipitated from the liquid by simple cooling down or addition of small amounts of water. As a drawback, it turned out that the extraction with both cyclic esters, but especially with ethylene carbonate, resulted in a high degree of depolymerization. The degree of depolymerization is highly dependent on temperature and time of the extraction (*79*).

A non-solvent-based method using a high-pressure homogenizer in the presence of SDS was also described. A maximum recovery yield of 98 % was achieved by homogenizing the cells at 400 kg/cm^2 in 5 % SDS solution (*80*).

Disruption of cells of osmophilic microbes in hypotonic medium

When highly osmophilic PHA production strains like *Haloferax mediterranei* are exposed to hypotonic media (distilled water), their cell envelopes become fragile. Under these conditions, the osmophilic cells burst, releasing all the cell components into the medium. Because of the considerable size and density of the PHA granules, they can easily be recovered by low speed centrifugation, sedimentation or filtration. If higher purity is demanded,

the obtained whitish crude sediment of PHA granules has to be washed further several times with detergents (*e.g.* SDS), which can break down impurities consisting of proteins and lipids. After drying, a fine powder made up of PHA with adequate purity is obtained, which can directly be used for polymer processing (*80*). Table 5 (*26, 50,56,73,74,76,79,81,82*) summarizes the major characteristics of known PHA isolation methods, distinguishing between the different isolation classes described above.

Conclusion

The article at hand demonstrated the necessity to switch to alternative polymeric materials. Strategies to overcome the major obstacles in the widespread applications of biopolyesters like PHA can be summarized as follows: the selection of the appropriate substrate first of all depends on the intended location of the PHA production plant, and on the resource quantities available. Moreover, the suitability of the raw material for low-cost storage is of high importance because such resources are very often available seasonally (*e.g.* straw, sugar cane, corn). Besides the carbon source, the availability of cheap complex nitrogen sources for effective and fast biomass production is advantageous. Adequate materials can be found in certain waste streams from agriculture like meat and bone meal, grass juices, or corn steep liquor. Wherever possible, industrial PHA production should be integrated into the existing structure of the industrial unit that generates waste or raw material to minimize production costs due to synergisms. A prime example of this strategy is the integration of PHA production into a sugar mill combined with ethanol production in Brazil. In this case, the in-house product sucrose acts as a carbon source, the total process energy (steam and electricity) is generated from surplus bagasse, and the extraction solvent is iso-pentanol available from the ethanol distillation unit.

Due to the enormous amounts available annually, lignocellulose-based wastes are likely to become the most important raw materials for future biotechnological production of polymers, chemicals and fuels. In order to make substantial progress in this field, increased know-how is required in the areas of microbiology, enzyme technology, and chemical engineering in order to overcome the still existing bottlenecks in the efficient conversion of the feedstock, especially during the upstream processing.

In the future, it will be indispensable to create data bases for agricultural feedstock and its side streams to document the range of variability in composition and quality. Such data bases can be obtained through a long-term monitoring and documentation of raw materials from different origins. Furthermore, it is desirable if the bioprocess itself is not sensitive against certain variability of the feedstock quality.

Improvements in the fermentation strategy by switching from discontinuous to continuous mode can be considered as the decisive step for achieving adequate productivities and to produce tailor-made products with constant qualities. In addition, continuous production provides a possibility to utilize inhibiting substrates at fine-tuned feeding rates.

Besides the raw material costs and the fermentation process itself, downstream processing is a decisive cost-

Table 5. Comparison of different PHA isolation methods

Method	Isolation class*	Method suitable for	Time demand	Investment costs	Costs for chemicals	Suitability for industrial scale	Recovery yields	Purity of isolated PHA	Impact on molecular mass of isolated PHA	Reference
'Classical' chloroform method	I	all strains	medium	low	high	no	high	high	low	*81*
'Agroferm method' (cyclic carbonates)	I	all strains	medium	low	medium	no	medium	high	high	*79*
'Brazilian method' *via* medium chain length alcohols	I	all strains	high	medium	available at the production plant as surplus product!	yes	high	low – medium	medium – high	*56*
Lactic acid ester method	I	all strains	medium – high	low	high	yes	low – medium	medium	medium – high	*73*
'Zeneca method' (enzymatic digestion of non-PHA cell material)	II	all strains	low	low	high	yes	high	low before refining	no	*74*
Alkaline digestion of non-PHA cell material	II	rec. *E. coli*	low	low	low	yes	medium	high	negligible	*50*
Hypochlorite method	II	all strains	medium	low	medium	no	medium	medium – high	medium – high	*76*
Cell disruption in hypotonic medium	III	highly osmophilic strains	low	low	no	yes	high	medium	no	*26,82*

*I: direct extraction of PHA from the biomass, II: digestion of the non-PHA cellular material, III: disruption of the cells of osmophilic microbes in hypotonic medium

-determining factor in biopolymer production. Depending on the PHA producing microorganisms and the demanded degree of product purity, a broad range of possibilities is available for PHA recovery and purification. Research in this field is quite advanced in terms of minimizing the required amounts of solvents and other cost intensive and/or hazardous compounds; what is missing are feasibility studies for the application on industrial scale.

A substantial progress towards a cost-efficient technology can be obtained by uniting the potential enhancements of each process step. In any case, the development of really efficient biopolymer production processes needs the narrow cooperation of experts from different scientific fields. Chemical engineers, microbiologists, enzymologists, polymer scientists, genetic engineers and experts in the fields of life cycle assessment (LCA) and cleaner production have to concentrate their special expertise and know-how in order to close the existing gaps between promising data from the laboratory scale and industrial realization.

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References

1. G. Braunegg, R. Bona, M. Koller, Sustainable polymer production, *Polym. Plast. Technol. Eng. 43* (2004) 1779–1793.
2. V. Gorenflo, A. Steinbüchel, S. Marose, M. Rieseberg, T. Scheper, Quantification of bacterial polyhydroxyalkanoic acids by Nile red staining, *Appl. Microbiol. Biotechnol. 51* (1999) 765–772.
3. D.K.Y. Solaiman, R.D. Ashby, A.T. Hotchkiss Jr, T.A. Foglia, Biosynthesis of medium-chain-length poly(hydroxyalkanoates) from soy molasses, *Biotechnol. Lett. 28* (2006) 157–162.
4. J. Cornibert, R.H. Marchessault, Conformational isomorphism. A general 2_1 helical conformation for poly(β-alkanoates), *Macromolecules, 8* (1975) 296–305.
5. G.N. Barnard, J.K.M. Sanders, Observation of mobile poly-(β-hydroxybutyrate) in the storage granules of *Methylobacterium* AM1 by *in vivo* ^{13}C-NMR spectroscopy, *FEBS Lett. 231* (1988) 16–18.
6. Y.B. Kim, R.W. Lenz, Polyesters from microorganisms, *Adv. Biochem. Eng. Biotechnol. 71* (2001) 51–79.
7. L.L. Wallen, W.K. Rhowedder, Poly-beta-hydroxyalkanoate from activated sludge, *Environ. Sci. Technol. 8* (1974) 576–579.
8. R.H. Findlay, D.C. White, Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*, *Appl. Environ. Microbiol. 45* (1983) 71–78.
9. A. Steinbüchel, H.E. Valentin, Diversity of bacterial polyhydroxyalkanoic acids, *FEMS Microbiol. Lett. 128* (1995) 219–228.
10. S.Y. Lee, Y. Lee, Metabolic engineering of *Escherichia coli* for production of enantiomerically pure (R)-(–)-hydroxycarboxylic acids, *Appl. Environ. Microbiol. 69* (2003) 3421–3426.
11. E. Shimamura, K. Kasuya, G. Kobayashi, T. Shiotani, Y. Shima, Y. Doi, Physical properties and biodegradability of microbial poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), *Macromolecules, 27* (1994) 878–880.
12. G.J. McCool, M.C. Cannon, PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in *Bacillus megaterium*, *J. Bacteriol. 183* (2001) 4235–4243.
13. H. Abe, I. Matsubara, Y. Doi, Y. Hori, A. Yamaguchi, Physical properties and enzymatic degradability of poly-(3-hydroxybutyrate) stereoisomers with different stereoregularities, *Macromolecules, 27* (1994) 6018–6025.
14. D.Y. Kim, K. Elbanna, N. Thakor, T. Lütke-Eversloh, A. Steinbüchel, Poly(3-mercaptopropionate): A non-biodegradable biopolymer?, *Biomacromolecules, 6* (2005) 897–901.
15. J. Agus, P. Kahar, H. Abe, Y. Doi, T. Tsuge, Molecular weight characterization of poly[(R)-3-hydroxybutyrate] synthesized by genetically engineered strains of *Escherichia coli*, *Polym. Degrad. Stabil. 91* (2006) 1138–1146.
16. A. Steinbüchel, S. Hein, Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms, *Adv. Biochem. Eng. Biotechnol. 71* (2001) 81–123.
17. A. Steinbüchel, T. Lütke-Eversloh, Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms, *Biochem. Eng. J. 16* (2003) 81–96.
18. M. Zinn, B. Witholt, T. Egli, Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate, *Adv. Drug Deliv. Rev. 53* (2001) 5–21.
19. S. Khanna, A.K. Srivastava, Recent advances in microbial polyhydroxyalkanoates, *Process Biochem. 40* (2005) 607–619.
20. S.F. Williams, D.P. Martin: Applications of PHAs in Medicine and Pharmacy. In: *Biopolymers, Vol. 4: Polyesters III – Applications and Commercial Products*, Y. Doi, A. Steinbüchel (Eds.), Wiley-VCH, Weinheim, Germany (2002) pp. 91–127.
21. Y. Doi: *Microbial Polyesters*, VCH Publishers Inc, Yokohama, Japan (1990) pp. 33–61.
22. Y. Poirier, C. Nawrath, C. Sommerville, Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants, *Biotechnology, 13* (1995) 142–150.
23. S.Y. Lee, Bacterial polyhydroxyalkanoates, *Biotechnol. Bioeng. 49* (1996) 1–14.
24. T.V. Ojumu, J. Yu, B.O. Solomon, Production of polyhydroxyalkanoates, a bacterial biodegradable polymer, *Afr. J. Biotechnol. 3* (2004) 18–24.
25. H. Son, S. Lee, Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from structurally unrelated single carbon sources by newly isolated *Pseudomonas* sp. EL-2, *Biotechnol. Lett. 18* (1996) 1217–1222.
26. F. Rodriguez-Valera, J.A.G. Lillo, Halobacteria as producers of polyhydroxyalkanoates, *FEMS Microbiol. Rev. 103* (1992) 181–186.
27. H.M. Alvarez, R. Kalscheuer, A. Steinbüchel, Accumulation of storage lipids in species of *Rhodococcus* and *Nocardia* and effect of inhibitors and polyethylene glycol, *Fett-Lipid, 99* (1997) 239–246.
28. H.E. Valentin, D. Dennis, Metabolic pathway for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) formation in *Nocardia corallina*: Inactivation of *mutB* by chromosomal integration of a kanamycin resistance gene, *Appl. Environ. Microbiol. 62* (1996) 372–379.
29. A. Akar, E.U. Akkaya, S.K. Yesiladali, G. Celikyilmaz, E.U. Cokgor, C. Tamerler, D. Orhon, Z.P. Cakar, Accumulation of polyhydroxyalkanoates by *Microlunatus phosphovorus* under various growth conditions, *J. Ind. Microbiol. Biotechnol. 33* (2006) 215–220.

30. M. Liebergesell, E. Hustede, A. Timm, A. Steinbüchel, R.C. Fuller, R.W. Lenz, H.G. Schlegel, Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria, *Arch. Microbiol. 155* (1991) 415–421.

31. M.F.A. Rodrigues, L.F. da Silva, J.G.C. Gomez, H.E. Valentin, A. Steinbüchel, Biosynthesis of poly(3-hydroxybutyric acid *co*-3-hydroxy-4-pentenoic acid) from unrelated substrates by *Burkholderia* sp., *Appl. Microbiol. Biotechnol. 43* (1995) 880–886.

32. R.J. Sánchez, J. Schripsema, L.F. da Silva, M.K. Taciro, J.G.C. Pradella, J.G.C. Gomez, Medium-chain-length polyhydroxyalkanoic acids (PHA_{mcl}) produced by *Pseudomonas putida* IPT 046 from renewable sources, *Eur. Polym. J. 39* (2003) 1385–1394.

33. G. Lefebvre, M. Rocher, G. Braunegg, Effects of low dissolved-oxygen concentrations on poly-(3-hydroxybutyrate--*co*-3-hydroxyvalerate) production by *Alcaligenes eutrophus*, *Appl. Environ. Microbiol. 63* (1997) 827–833.

34. J.S. Kim, B.H. Lee, B.S. Kim, Production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) by *Ralstonia eutropha*, *Biochem. Eng. J. 23* (2005) 169–174.

35. Y.H. Lee, M.S. Kang, Y.M. Jung, Regulating the molar fraction of 4-hydroxybutyrate in poly(3-hydroxybutyrate-4-hydroxybutyrate) biosynthesis by *Ralstonia eutropha*, using propionate as a stimulator, *J. Biosci. Bioeng. 89* (2000) 380–383.

36. N. Narayanan, P.K. Roychoudhury, A. Srivastava, L(+) lactic acid fermentation and its product polymerization, *Electron. J. Biotechnol. 7* (2004) 167–178.

37. H.O. Kim, Y.J. Wee, J.N. Kim, J.S. Yun, H.W. Ryu, Production of lactic acid from cheese whey by batch and repeated batch cultures of *Lactobacillus* sp. RKY2, *Appl. Biochem. Biotechnol. 131* (2006) 694–704.

38. R.E. Drumright, P.R. Gruber, D.E. Henton, Polylactic acid technology, *Adv. Mater. 12* (2000) 1841–1846.

39. Y.J. Wee, J.N. Kim, H.W. Ryu, Biotechnological production of lactic acid and its recent applications, *Food Technol. Biotechnol. 44* (2006) 163–172.

40. S. Taguchi, M. Yamada, K. Matsumoto, K. Tajima, Y. Satoh, M. Munekata, K. Ohno, K. Kohda, T. Shimamura, H. Kambe, S. Obata, A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme, *PNAS, 105* (2008) 17323–17327.

41. K. Sudesh, T. Iwata, Sustainability of biobased and biodegradable plastics, *Acta Hydrochim. Hydrobiol. 36* (2008) 433–442.

42. G. Braunegg, G. Lefebvre, K.F. Genser, Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and engineering aspects, *J. Biotechnol. 65* (1998) 127–161.

43. D.K.Y. Solaiman, R.D. Ashby, T.A. Foglia, W.N. Marmer, Conversion of agricultural feedstock and coproducts into poly(hydroxyalkanoates), *Appl. Microbiol. Biotechnol. 71* (2006) 783–789.

44. A.A. Khardenavis, M.S. Kumar, S.N. Mudliar, T. Chakrabarti, Biotechnological conversion of agro-industrial wastewaters into biodegradable plastic, poly β-hydroxybutyrate, *Bioresour. Technol. 98* (2007) 3579–3584.

45. M. Koller, R. Bona, G. Braunegg, C. Hermann, P. Horvat, M. Kroutil, J. Martinz, J. Neto, L. Pereira, P. Varila, Production of polyhydroxyalkanoates from agricultural waste and surplus materials, *Biomacromolecules, 6* (2005) 561–565.

46. R.D. Ashby, D.K.Y. Solaiman, T.A. Foglia, Bacterial poly-(hydroxyalkanoate) polymer production from biodiesel co--product stream, *J. Polym. Environ. 12* (2004) 105–112.

47. M. Koller, P. Hesse, R. Bona, C. Kutschera, A. Atlić, G. Braunegg, Potential of various archae- and eubacterial strains as industrial polyhydroxyalkanoate producers from whey, *Macromol. Biosci. 7* (2007) 218–226.

48. M. Koller, R. Bona, C. Hermann, P. Horvat, J. Martinz, J. Neto, P. Varila, G. Braunegg, Biotechnological production of poly(3-hydroxybutyrate) with *Wautersia eutropha* by application of green grass juice and silage juice as additional complex substrates, *Biocatal. Biotransform. 23* (2005) 329–337.

49. H. Zhang, V. Obias, K. Gonyer, D. Dennis, Production of polyhydroxyalkanoates in sucrose-utilizing recombinant *Escherichia coli* and *Klebsiella* strains, *Appl. Environ. Microbiol. 60* (1994) 1198–1205.

50. J.I. Choi, S.Y. Lee, Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant *Escherichia coli* by simple digestion with chemicals, *Biotechnol. Bioeng. 62* (1999) 546–553.

51. T. Fukui, Y. Doi, Efficient production of polyhydroxyalkanoates from plant oils by *Alcaligenes eutrophus* and its recombinant strain, *Appl. Microbiol. Biotechnol. 49* (1998) 333–336.

52. P.Y. Bourque, Y. Pomerleau, D. Groleau, High-cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: Production of high-molecular-mass PHB, *Appl. Microbiol. Biotechnol. 44* (1995) 367–376.

53. G. Braunegg, M. Koller, P. Varila, C. Kutschera, R. Bona, C. Hermann, P. Horvat, J. Neto, L. Pereira: Production of Plastics from Waste Derived from Agrofood Industry. In: *Renewable Resources and Renewable Energy: A Global Challenge*, M. Graziani, P. Fornasiero (Eds.), CRC Press, Taylor&Francis Group, Boca Raton, FL, USA (2007) pp. 119–135.

54. R. Kumar, S. Singh, O.V. Singh, Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives, *J. Ind. Microbiol. Biotechnol. 35* (2008) 377–391.

55. D. Peters, Carbohydrates for fermentation, *Biotechnol. J. 1* (2006) 806–814.

56. R.V. Nonato, P.E. Mantelatto, C.E.V. Rossell, Integrated production of biodegradable plastic, sugar and ethanol, *Appl. Microbiol. Biotechnol. 57* (2001) 1–5.

57. G. Braunegg, G. Lefebvre, G. Renner, A. Zeiser, G. Haage, K. Loidl-Lanthaler, Kinetics as a tool for polyhydroxyalkanoate production optimization, *Can. J. Microbiol. 41* (1995) 239–248.

58. G. Du, J. Chen, J. Yu, S. Lun, Continuous production of poly-3-hydroxybutyrate by *Ralstonia eutropha* in a two-stage culture system, *J. Biotechnol. 88* (2001) 59–65.

59. N. Koyama, Y. Doi, Continuous production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Alcaligenes eutrophus*, *Biotechnol. Lett. 17* (1995) 281–284.

60. S. Aiba, A.E. Humphrey, N.F. Millis: *Biochemical Engineering*, Academic Press, Inc., New York, NY, USA (1973).

61. M. Zlokarnik, Suitability of stirrers for homogenization and mixing of liquid mixtures, *Chem. Ing. Technol. 39* (1967) 539–548 (in German).

62. R. Hartmann, R. Hany, E. Pletscher, A. Ritter, B. Witholt, M. Zinn, Tailor-made olefinic medium-chain-length poly-[(*R*)-3-hydroxyalkanoates] by *Pseudomonas putida* GPo1: Batch versus chemostat production, *Biotechnol. Bioeng. 93* (2001) 737–746.

63. R. Hartmann, R. Hany, T. Geiger, T. Egli, B. Witholt, M. Zinn, Tailored biosynthesis of olefinic medium-chain-length poly[(*R*)-3-hydroxyalkanoates] in *Pseudomonas putida* GPo1 with improved thermal properties, *Macromolecules, 37* (2004) 6780–6785.

64. B.A. Ramsay, K. Lomaliza, C. Chavarie, B. Dubé, P. Bataille, J.A. Ramsay, Production of poly-(β-hydroxybutyric-*co* -β-hydroxyvaleric) acids, *Appl. Environ. Microbiol. 56* (1990) 2093–2098.

65. S.T. Yu, C.C. Lin, J.R. Too, PHBV production by *Ralstonia eutropha* in a continuous stirred tank reactor, *Process Biochem. 40* (2005) 2729–2734.

66. M. Zinn, H.U. Weilenmann, R. Hany, M. Schmid, T. Egli, Tailored synthesis of poly([*R*]-3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHB/HV) in *Ralstonia eutropha* DSM 428, *Acta Biotechnol. 23* (2003) 309–316.

67. G. Mothes, J.U. Ackermann, Synthesis of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) with a target mole fraction of 4-hydroxybutyric acid units by two-stage continuous cultivation of *Delftia acidovorans* P4a, *Eng. Life Sci. 5* (2005) 58–62.

68. K. Jung, W. Hazenberg, M. Prieto, B. Witholt, Two-stage continuous process development for the production of medium-chain-length poly(3-hydroxyalkanoates), *Biotechnol. Bioeng. 72* (2001) 19–24.

69. G.Q. Chen, G. Zhang, S.J. Park, S.Y. Lee, Industrial scale production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), *Appl. Microbiol. Biotechnol. 57* (2001) 50–55.

70. M. Terada, R.H. Marchessault, Determination of solubility parameters for poly(3-hydroxyalkanoates), *Int. J. Biol. Macromol. 25* (1999) 207–215.

71. J. Choi, S.Y. Lee, Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation, *Appl. Microbiol. Biotechnol. 51* (1999) 13–21.

72. G. Braunegg, R. Bona, F. Schellauf, E. Wallner, Polyhydroxyalkanoates (PHAs): Sustainable biopolyester production, *Polimery, 47* (2002) 13–18.

73. K. Metzner, M. Sela, J. Schaffer, Agents for extracting polyhydroxyalkane acids. *WO patent 9708931* (1997).

74. P.A. Holmes, G.B. Lim, Separation process. *US patent 4910145* (1990).

75. K. Yasotha, M.K. Aroua, K.B. Ramachandran, I.K.P. Tan, Recovery of medium-chain-length polyhydroxyalkanoates (PHAs) through enzymatic digestion treatments and ultrafiltration, *Biochem. Eng. J. 30* (2006) 260–268.

76. E. Berger, B.A. Ramsay, J.A. Ramsay, C. Chavarie, G. Braunegg, PHB recovery by hypochlorite digestion of non-PHB biomass, *Biotechnol. Technique, 3* (1989) 227–232.

77. B. Ramsay, J. Ramsay, E. Berger, C. Chavarie, G. Braunegg, Separation of poly-beta-hydroxyalkanoic acid from microbial biomass. *US patent 5110980* (1992).

78. M. Kim, K.S. Cho, H.W. Ryu, E.G. Lee, Y.K. Chang, Recovery of poly(3-hydroxybutyrate) from high cell density culture of *Ralstonia eutropha* by direct addition of sodium dodecyl sulphate, *Biotechnol. Lett. 25* (2003) 55–59.

79. R.M. Lafferty, E. Heinzle, Cyclic carbonic acid esters as solvents for poly-(β-hydroxybutyric acid. *US patent 4101533* (1978).

80. M.S. Ghatnekar, J.S. Pa, M. Ganesh, Production and recovery of poly-3-hydroxybutyrate from *Methylobacterium* sp. V49, *J. Chem. Technol. Biotechnol. 77* (2002) 444–448.

81. I. Chodak: Polyhydroxyalkanoates: Properties and Modification for High Volume Applications. In: *Degradable Polymers: Principles and Applications*, G. Scott (Ed.), Kluwer Academic Publishers, Dordrecht, the Netherlands (2002) pp. 295–319.

82. E.A. Muñoz-Escalona, F. Rodríguez-Valera, A. Marcilla Gomis, Procedure for the extraction of polyhydroxyalkanoates from halophilic bacteria which contain them. *European patent 0622462* (1994).