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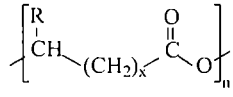
# Polyhydroxyalkanoates (PHAs): sustainable biopolyester production

**Summary** — Microbial polyhydroxyalkanoate (PHA) reserve polymers are interesting polyesters that can be sustainably produced by biotechnological means from a variety of renewable substrates. By feeding cosubstrates to selected cultures of prokaryotic microorganisms composition, structure and therefore physical properties of the PHAs can be influenced during their biotechnological production. Cheap substrates stemming from agricultural waste and surplus streams have to be used in order reduce product costs to a level similar to that of conventional plastics. Continuous production in combinations of a stirred tank reactor for microbial growth and a tubular plug flow reactor for PHA accumulation in the microbial biomass form the ideal production system. PHA is accumulated within the producing cells and has to be extracted. A system of chloroform, ethanol and water allows reusing the extracting solvent chloroform without prior redistillation.
**Key words**: polyhydroxyalkanoates (PHAs), production, sustainability.

## PHAs STRUCTURE — GENERAL REMARKS

Polyhydroxyalkanoates [general formula — see (I)] are polyesters produced and stored intracellularly in high concentrations as reserve materials for carbon and energy by a wide range of prokaryotic microorganisms


(I)

under imbalanced nutritional conditions [1, 2]. A variety of such materials can be produced in bioreactors by feeding of different renewable carbon sources and precursors, leading to copolyesters having differing physical properties. Due to the fact that the prices for the main carbon sources are decisive for the production costs of such bulk products, only cheap substrates can be used in such production processes for high volume low cost materials. Depending of the resources available in different countries, mainly agricultural waste and surplus materials containing sugars, alcohols, or organic acids are interesting raw materials. In certain regions, petrol-derived carbon sources (*e.g.* methanol) are of interest as carbon sources as well.

Beside the homopolyester poly(*R*)-3-hydroxybutanoate, consisting of 3-hydroxybutanoate (3HB) only two main types of copolyesters can be formed by different microorganisms [3]. The first type of PHAs always contains $C_3$ units in the polymer backbone, but the side chains can contain H, methyl or ethyl groups if it is prepared with microorganisms like *Ralstonia eutropha*, or propyl to nonyl groups — if the copolyester is prepared with *Pseudomonas oleovorans*. In the latter case, branching [4], double bonds [5], epoxides [6], and aromatic structures [7] can be also introduced into the side chain. Furthermore copolyesters containing ω-chloroalkanoates (F, Cl, Br) can be produced [8—10]. In the case of *P. oleovorans* and other strains from the group of fluorescent pseudomonas PHA formation occurs only when the organisms are grown either with fatty acids (butanoate to hexadecanoate) or with alkanes (hexane to dodecane). Doi [11] recently reported about synthesis of a copolyester consisting of 3-hydroxybutyrate and 3-hydroxyhexanoate by *Aeromonas cavei*, and Chen [12] isolated a bacterium from oil-contaminated earth able to synthesize the same polyester when fed with glucose and laurylic acid. This copolyester shows an extremely high extension needed to break.

The second type of PHA is polyester with short side chain, containing hydrogen, methyl, or ethyl groups in these chains, and having $C_3$, $C_4$, and $C_5$ units in the backbone of the polymer [13, 14]. Carbohydrates, alcohols, and low fatty acids are typical substrates for growth and PHA formation for these prokaryotic microorganisms. In most cases, cosubstrates have to be fed to the producing cultures as precursors for copolyester formation [14, 15]. Typical precursors that have been used are propionate, valerate, or 1,4-butanediol, leading to analogues of 3HB such as 4-, and 5-hydroxyalkanoates.

Formation of random copolyesters results in many physical changes in the PHAs, including liquid-crystalline-amorphous forms, and a variety of piezoelectric, thermoplastic, elastomeric and other properties [16].

The "mixed" polyesters formed depend on the organisms used to produce them, and on the carbon sources and polyester precursors. For PHAs formed by prokaryotes, the main physiological role accepted is that the polyesters function as carbon and energy reserve materials. When growth is limited by exhaustion of *e.g.* nitrogen, phosphate, sulfur, oxygen in the nutritional broth, excess carbon is channeled into PHAs, leading to polyesters with molecular weights of up to about 3.4 MDa [17].

Recently, other physiological roles have been recognized for PHAs. Relatively small molecular weight PHAs (up to about 35 KDa) are incorporated into membranes and their tertiary helical structures (backbone inside), such as ion pores, are putatively used for transport of ions into and out of the cells [18—20].

PHAs can be applied as a biodegradable substitute to fossil fuel plastics that can be produced from renewable raw materials such as saccharides, alcohols and low-molecular-weight fatty acids. They are completely degradable to carbon dioxide and water through natural microbiological mineralization. Consequently, neither their production nor their use or degradation have a negative ecological impact. By maintaining a closed cycle of production and re-use, PHAs can enable at least part of the polymer-producing industry to switch from ecologically harmful end-of-the-pipe production methods towards sounder technologies.

## PHA METABOLISM AND PHA PRODUCING MICROORGANISMS

When bacteria able to store PHAs are grown in imbalanced media, certain media components like the assimilable nitrogen source, *e.g.* $(NH_4)_2SO_4$, or other are depleted during microbial growth. In such cases, synthesis of important cell constituents such as proteins, DNA, RNA *etc.*, cannot take place, and acetyl-CoA derived from incomplete oxidation of carbon compounds will now be fed into the tricarbonic acid cycle at a lower rate than during the normal growth phase. This key component will now enter the metabolic routes of PHA synthesis, and depending on the availability of other acyl-CoA derivatives, acetoacetyl-CoA (or other homologues) will be formed by a condensation reaction. Acetoacetyl-CoA is reduced to *R*-3-hydroxybutyryl-CoA, and further used for a polymerization to form the homopolyester — poly(*R*)-3-hydroxybutyrate — or copolyesters that will be stored by the bacteria in form of globular granules.

When growth limitation for PHA accumulating cells is abolished (*e.g.* by addition of assimilable nitrogen to the production medium), PHA stored in the cells is depolymerized to the monomers *via* oligomers as intermediates. These can be further metabolized to $CO_2$ and water, producing adenosintriphosphate (energy for the cell) at the same time. The same is true for pure PHA extracted from bacterial cells. The polyester can be degraded by extracellular depolymerases that can be produced by a high number of prokaryotic and eukaryotic microorganisms.

PHAs can be synthesized by many different prokaryotic microorganisms; Table 1 shows PHA accumulating genera of bacteria. Depending on the metabolic capacities of these microorganisms, many different carbon sources can be used for a PHA production process; the combination of microorganism, carbon source, precursor, and limiting substrate are decisive for the polymer quality produced. For enrichment of high productivity strains continuous culture methods are available. Many of the genera shown in Table 1 are described as PHA storing organisms, but in most cases no data are available about possible copolyester compositions, growth and production kinetics.

**Table 1. PHA accumulating genera of microorganisms**

| | | |
|---|---|---|
| Acinetobacter | Escherichia [a] | Paracoccus |
| Actinomycetes | Ferrobacillus | Pedomicrobium |
| Alcaligenes [a,b] | Gamphospheria | Photobacterium |
| Aphanothece [a] | Haemophilus | Pseudomonas [a,b] |
| Aquaspirillum | Halobacterium [a] | Rhizobium [a,b] |
| Asticcaulus | Hyphomicrobium | Rhodobacter |
| Azomonas | Lamprocystis | Rhodococcus [b] |
| Azospirillum | Lampropedia | Rhodopseudomonas |
| Azotobacter [a] | Leptothrix | Rhodospirillum [b] |
| Bacillus [a,b] | Methanomonas | Sphaerotilus [a] |
| Beggiatoa | Methylobacterium [b] | Spirillum |
| Beijerinckia [b] | Methylocystis | Spirulina |
| Beneckea | Methylomonas | Stella |
| Caryophanon | Methylovibrio | Streptomyces |
| Caulobacter | Micrococcus | Syntrophomonas |
| Chlorofrexeus | Microcoleus | Thiobacillus |
| Chlorogloea | Microcystis | Thiocystis |
| Chromatium | Moraxella | Thiodictyon |
| Chromobacterium | Mycoplana [a] | Thiopedia |
| Clostridium | Nitrobacter | Thiosphaera |
| Corynebacterium [b] | Nitrococcus | Vibrio |
| Derxia [b] | Nocardia [a,b] | Xanthobacter |
| Ectothiorhodospira | Oceanospirillum | Zoogloea [a] |

[a] — Detailed knowledge about growth and production kinetics available.
[b] — Accumulation of copolyesters known.

If copolyesters containing different comonomers are the goal of the PHA production process, precursors have to be fed to the medium during production phase of the microorganisms. Chemical nature and concentrations of these precursors have to be controlled carefully, in order not to reach an inhibitory or even toxic level, and to guarantee a wanted level of comonomer in the copolyesters. Presently, more than 150 precursors are known, leading to a wide range of copolyesters differing in their

physical behavior. Unfortunately, many of them are too expensive to be used in industrial production, and normally also the comonomer yields from the precursors are rather low, if no special precautions are taken in consideration (*e.g.* dissolved oxygen partial pressure during PHA accumulation). Examples of such precursors for *Ralstonia eutropha* are given in Table 2.

**Table 2. Examples for precursors added to production media in adequate concentrations during PHA accumulation phase for *Ralstonia eutropha***

| Precursor | Copolyester |
|---|---|
| Propionate | Poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate |
| γ-Butyrolactone | Poly-3-hydroxybutyrate-*co*-4-hydroxybutyrate |
| 1,4-Butanediol | Poly-3-hydroxybutyrate-*co*-4-hydroxybutyrate |
| 4-Hydroxybutyrate | Poly-3-hydroxybutyrate-*co*-4-hydroxybutyrate |

A limited number of strains is known store copolyesters without addition of special precursors. Some examples are shown in Table 3.

**Table 3. Examples for copolyester production without precursor addition**

| Strain | Copolyester formed |
|---|---|
| *Alcaligenes* sp. SH-69 | Poly-3-hydroxybutyrate-*co*-3-hydroxy-valerate from C-sources like glucose, sucrose, sorbitol, mannitol, and glutamate |
| *Pseudomonas cepacia* | Poly-3-hydroxybutyrate-*co*-3-hydroxy-4-pentenoate from C-sources like gluconate and sucrose |
| *Pseudomonas* sp. (NCIMB 40135) | Copolyester from 3-hydroxydecanoate (mainly) and 3-hydroxyoctanoate from substrates like acetate, glycerol, lactate, succinate, glucose, gluconate, or *n*-octanoate |

## CARBON SOURCES FOR PHA PRODUCTION

As was mentioned above, prices for raw materials for PHA production play a crucial role for the economy of a PHA production process. Roughly, it can be stated that in an aerobic process for production of a bulk chemical such as PHAs, 50% of the production costs originate from the costs for the carbon source used. In Table 4, "classical" carbon sources used in defined media are shown, but it has to be mentioned that PHA production in such media in most cases is too expensive for an industrial process, and often microbial growth rates polyester productivity are too low. Nevertheless, experiments for PHA production using such defined media are important for studies of production kinetics, PHA yields attained and other factors, depending from a specific carbon source to be used.

Rather complex growth and production media have to be used for future high volume industrial PHA production. Table 5 shows a list of cheap renewable resources that, beside a main carbon source (*e.g.* carbohydrates), always contain other components that might serve as additional carbon or nitrogen sources for growing or PHA producing strains. Depending on the concentrations of these sources (Table 6), such media have to be supplemented in order to keep the balance between the components in ranges suitable for the producing mi-

**Table 4. "Classic" substrates in defined media**

| Substrate | Examples of useful organism |
|---|---|
| Carbohydrates (glucose, fructose, sucrose) | *Ralstonia* sp., *Pseudomonas* sp., *Azotobacter* sp., *Rhizobium* sp., |
| Alcohols (methanol) | *Methylobacter* sp., *Methylomonas* sp., *Mycoplana* sp., |
| Alkanes ($C_6$—$C_{12}$) | *Pseudomonas Oleovorans*, |
| Organic acids (butyrate, valerate) | *Ralstonia* sp., *Alcaligenes* sp., |
| Organic acids ($C_6$—$C_{12}$) | *Pseudomonas Oleovorans* |

**Table 5. PHA-Production from cheap renewable resources for sustainable process development in complex growth and production media**

| Carbohydrates | Molasses |
|---|---|
| | Starch and starch hydrolysates (maltose) |
| | Lactose from whey |
| | Cellulose hydrolysates (*e.g.* reject fiber wastes from the paper industry after hydrolysis and ion exchange for heavy metal removal) |
| Alcohols | Wastes from biodiesel production: methanol plus glycerol |
| Fats and oils | Plant and animal waste, food waste |
| Organic acids | Lactic acid from dairy industry |

**Table 6. Average composition of complex carbon sources for PHA production**

**A) Molasses and Green Syrup**

| Carbohydrates, % | Beet molasses | Cane molasses | Green syrup |
|---|---|---|---|
| Sucrose | 48.5 | 33.4 | 50.5 |
| Inverted sugar | 2.5 | 21.2 | 3.8 |
| Raffinose | 2.1 | | 1.1 |

**B) Whey**

| Compounds, % | Sweet whey | Fermented whey | Whey permeate |
|---|---|---|---|
| Lactose | 4.7—4.9 | 4.5—4.9 | 23 |
| Lactic acid | traces | 0.5 | — |
| Protein (*N*-compounds) | 0.75—1.1 | 0.45 | 0.75 |
| Fats | 0.15—0.2 | traces | — |
| Salts | 0.6 | 0.6 | 2 |
| Ashes | — | — | 3.25 |
| Solids, total | *ca.* 7 | 6—7 | *ca.* 27 |

**C) Sulfite Liquor**

| Type of wood | Sugars, total, % |
|---|---|
| Soft wood | 2—3.5 |
| Hard wood | 3—4 |

croorganisms. In many cases, practical difficulties can arise from the fact that composition of such media components are not constant year to year, and also the costs for these raw materials can change considerably. Therefore, a process based on a certain raw material has to adapt, and the quality control of raw materials is important. Furthermore, the PHA production process has to be kept monoseptic (only the producing bacterial strain is propagated in the bioreactor). Thus, media and reactors have to be sterilized and no degradation of media components may occur during this step of the upstream processing.

Beside the carbon sources shown, whole plant utilization of crops might be an alternative way to derive cheap raw materials for PHA production. One of such possibilities is shown in Fig. 1 for the utilization of corn (Zea mais) as a whole plant. Especially the non-starch part of the plant is not used today, even though during corn harvesting its biomass could easily be harvested. Additionally to the production of starch, the plant could be a reservoir for cellulose, hemicellulose and lignin. Cellulose and hemicelluloses can be hydrolyzed to hexoses and pentoses that later may be used as raw materials for PHA production. Furthermore, corn fibers can be produced as fillers, and an eventual surplus of the crop material could be used for energy production. A similar approach can be seen in Brazil, where surplus bagasse is used for energy production in the production process for sucrose, and cheap surplus energy can be used for other biotechnological processes, *e.g.* PHA production.

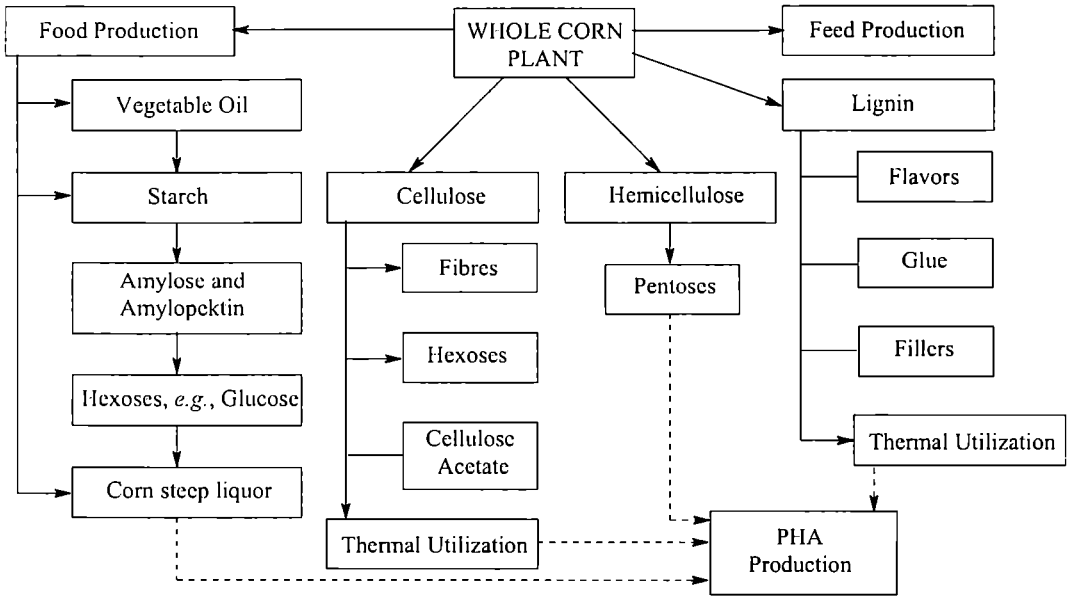



*Figure 1. Whole plant utilization of corn: food and feed production plant residuals of Zea mais can either directly or indirectly be used for PHA production*

## PHA PRODUCTION KINETICS AND ITS INFLUENCE ON PROCESS DESIGN

Own findings and those reviewed from the literature point out the existence of three distinct types of growth and PHA accumulation behavior, each one typified here by one or more microorganism-carbon source combination:

— PHA synthesis occurs in association with growth (*e.g. A. latus* with sucrose): autocatalytic PHA accumulation process

— PHA synthesis occurs in partial association with growth (*e.g. R. eutropha* $G^{+3}$ with glucose, *A. latus* with glucose): PHA accumulation process partially autocatalytic, but mainly normal catalytic

— PHA is hyperproduced after a carbon starvation period (*e.g. Pseudomonas* 2 F with glucose): short autocatalytic PHA accumulation followed by normal catalysis.

In principle, these three behaviors can be exploited for PHA production in batch culture, but due to its higher productivity, a continuous production process is of higher commercial interest, especially for strains with a high maximum specific growth rate. To prove this point, the overall productivity of a batch system will be compared to that of a continuous culture in the following manner [21]:

$$\frac{Pr_{CSTR}}{Pr_{DSTR}} = \ln\frac{X_e}{X_i} + t_0\mu_{max} \qquad (1)$$

where: $Pr_{CSTR}$ *and* $Pr_{DSTR}$ *— productivities of a continuous stirred-tank reactor (CSTR) and a discontinuous stirred-tank reactor (DSTR), respectively,* $X_e$ *— maximum biomass concentration,* $X_i$ *— initial biomass concentration;* $t_0$ *— period of time between the end of a production run and the start of the next one,* $\mu_{max}$ *— the maximal specific growth rate of the producing strain.*

Switching to a continuous process can in many processes enhance productivity by a factor between 5 and 10. The use of values of $\mu_{max}$ for *A. latus* and *R. eutropha* $G^{+3}$ and a maximum biomass concentration of 30 $gL^{-1}$ in

Eq. 1 gives a productivity ratio of 8.2 with *A. latus* and 5.25 with *R. eutropha* if $t_0$ is set to a low 10 h. This means that for a fixed desired amount of product per unit of time, the bioreactor volume can be substantially reduced if a continuous culture is chosen in favor of a batch process. From an engineering point of view, reactor performance would also be easier to control, as lower fermentor volumes lead to less segregation through better mixing at inferior energy expenditure [22].

But, if a continuous process is considered, the issue of kinetics must be addressed. Data from our experiments suggest that a further increase of productivity for PHA accumulation is possible, if a reactor system fitting kinetic demands of PHA accumulation is chosen. This leads to the proposition that in a multi-stage continuous PHA-production process use of a plug-flow tubular reactor (PFTR), in which Reynolds numbers are large, brings substantial increases in productivity when compared to a system consisting of a CSTR only. Support of this assertion comes from the works of Levenspiel [23], where mean residence times for the two types of reactors are compared under the restriction of a desired goal. Using the results attained with data coming from experiments with *Alcaligenes latus* and *Ralstonia eutropha* this allows to conclude that: In order to have the same productivity, a PFTR used as a second step would require a volume of 19.8% for *Alcaligenes latus* if compared with a CSTR, in the case of *Ralstonia eutropha* the effect is even higher, so that a PFTR would only need 11.4% compared to a CSTR as a PHA production bioreactor.

The CSTR-PFTR arrangement not only guarantees maximum productivity, but also minimizes cosubstrate loss, and might be used for enhancing product quality, since very narrow residence time distributions (and therefore uniform cell population) are characteristic of plug-flow tubular reactors [3]. Instead of a PFTR as a second step, a cascade of CSTRs can be used alternatively, allowing additional control of the cell-produced PHA in all reactors in the cascade individually.

## DOWNSTREAM PROCESSING FOR PHA ISOLATION

Isolation of the PHA from the microbial biomass is another the cost factor, besides the carbon source and the fermentation process. This step can be performed either by extracting the polyester from the separated microbial biomass, or by solubilization of the non PHA biomass. A method that has been described quite often in literature comprises PHA extraction with hot chloroform from dried microbial biomass and precipitation of the polyester by simply adding ethanol. Even though such a procedure is quite simple, reutilization of the extracting solvent is not so easy, because separation of chloroform and ethanol by distillation is energetically quite costly.

Therefore, we have tried to simplify this step of solvent reutilization by making use of the advantages of the chloroform-ethanol-water system for such separation (Fig. 2). As can be seen from Fig. 2, these three components can form different phases depending on their relative concentrations. Thus, it is possible to separate $CHCl_3$ from the chloroform/ethanol mixture by adding water: two phases are formed; their composition is shown in Table 7.

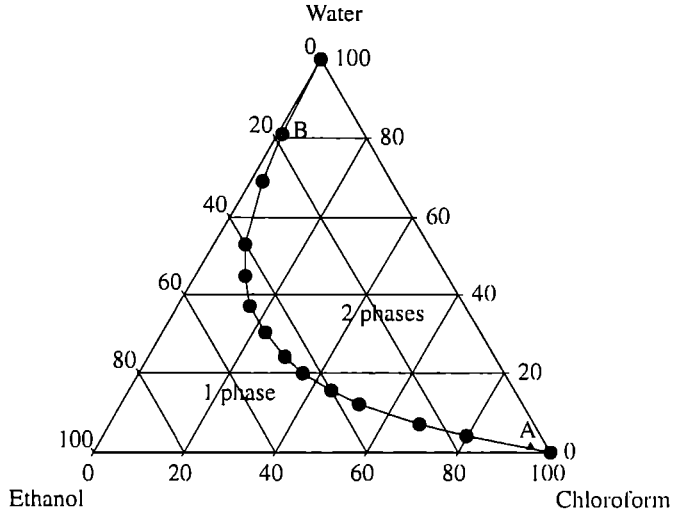



*Figure 2. Phase diagram for the water — ethanol — chloroform system*

**T a b l e 7. Composition of the Phases A and B for chloroform separation**

| Composition / Phase | Chloroform % v/v | Ethanol % v/v | Water % v/v |
|---|---|---|---|
| Lower phase (A) | 95 | 3.7 | 1.3 |
| Upper phase (B) | 1.0 | 18 | 81 |

It has to be stated that even better yields can be achieved, if the process is run continuously. Phase A can be reused for PHA extraction directly and, optionally, water content of phase A can be removed by addition of water-free $Na_2SO_4$. Neither is removal of ethanol needed, because technical grade chloroform always contains about 1% of ethanol for stabilization reasons and a low energy consuming membrane process can replace distillation for component separation in phase B.

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