Mini Review

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Polyhydroxyalkanoate production in biofermentor monitored through biosensor application

Abstract

Background

Polyhydroxyalkanoates (PHAs) synthesized by bacteria are the primary source for production of biodegradable bioplastics. PHAs can be produced by microbial cultures fed with renewable feed stocks. PHAs are a great opportunity for the polymer industry. While some bacterial species produce mainly PHB homopolymers consisting only of 3HB monomers, other species can synthesize various PHAs, depending on availability of intermediate precursors. The enzymes synthesizing the polymers, PHA syntheses are encoded by the PhaC genes. Differences in primary and secondary structure determine the capacity to accommodate various hydroxyalkanoate substrates and to form short-chain-length or medium-chain-length PHAs. In this review we discuss the enzyme structure and differences at the base of the characteristic polymers formed. Finally, we review innovative methods applied to biofermentor and on-line fermentation to monitor the production of PHAs, finalized to optimization of fermentation conditions, and the extraction methods available.

Keywords: bacteria, fermentation, feedstock, polyhydroxyalkanoates (PHA), biosensor methods

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Introduction

Polyhydroxyalkanoates (PHAs), synthesized by Gram-negative and Gram-positive bacteria, as well as Archaea and cyanobacteria, are the primary source for production of biodegradable bioplastics. Applications of PHAs are very different and assorted, from coatings, bottles, packaging films, in medical devices and for drug delivery, conductive biopolymers, high-tech electronics, car components, lightweight structural elements in buildings, textile elastomers and fibres, disposable materials, performance additives, transparent films, fertilizer mulches in soil application.[1] Poly(3-hydroxybutyrate) (P3HB) is the basic polymer for biodegradable bioplastics, is tough, brittle, fragile, stiff, with low elongation ability, and a break point below 15%. During ageing, PHB tends to re crystallize, and its mechanical properties change with time. PHB is made less stiff through plasticizers addition, and can change mechanical property by adding fibers and compounding materials. Polyhydroxyalkanoate (PHA) polymers contain various 3-hydroxyalkanoate monomers, from 3-hydroxypropionate (3HP), 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx), 3-hydroxyheptanoate (3HHp), up to 3-hydroxydodecanoate (3HDD), and in some circumstances also 4-hydroxybutyrate (4HB) and 5-hydroxyvalerate (5HV). PHAs are synthesized in microbial cultures, fed with renewable feed stocks. PHAs are insoluble in water, UV resistant, do not hydrolyze, tend to be degraded in acid and basic solutions, are soluble in chloroform and chlorinated solvents, are biocompatible biologically, being present in human fluids and tissues, are degraded in anaerobiosis, and are not "sticky" during melting, compared to other polymers. Short-chain-length (scl) PHA are formed by 3-hydroxy-C3 to C6 alkane units.[2–11] Medium-chain-length (Mcl) PHAs (i.e. composed of 3-hydroxy- C6 to C12 -alkanes) have optimum elongation property, higher break point and lower melting temperature in respect to scl-PHAs.

Homopolymers, copolymers, and grafted polymers

While some bacterial species produce mainly PHB homopolymers, other species can synthesize PHAs formed by various 3-hydroxyalkanoates, when the required intermediate precursors are provided through engineered metabolic pathways or as feedstock,[1] optimized substrate availability (by successive addition of precursors), and use of PHA syntheses with broader substrate requirement, with ability to locate larger hydroxyalkanoate monomers within the substrate binding pocket. The structure and properties of the polymers are largely influenced by the ratio of 3-hydroxyalkanoate monomers in respect to 3-HB. 3-hydroxyvalerate and other co-monomers improve the elasticity of PHAs. The synthesis of copolymers is a frequent strategy to adapt PHAs to the required properties, with improvement of flexibility of bioplastics, that will show lower glass transition temperature (Tg) and melting temperature (Tm).[2] PHA homopolymers can have great size, as in high molecular weight (hmw-PHB). Other produced polymers may contain also 4-hydroxybutyrate (P4HB), 3-hydroxy-5-phenylvalerate P(3HPhV), 3-hydroxy-6-phenylhexanoate (3H6PhHs), as in functionalized scl- and mcl- PHAs.

P3HB-4HB polymers are conventional thermoplastic material exploited in packaging. These bioplastics have high tensile strength and high elongation until breakpoint. PHB copolymers containing 3-hydroxyvalerate (P-3HB-co-3HV) have very good mechanical property, being elastic, with good tensile strength, low melting point, and low degree of crystallinity. The thermal, mechanical, and barrier properties of PHBV with different 3HV content, and with various molecular weight, are excellent. The presence of 3HV in the copolymer supports the production process, presents good

melting stability at temperatures below 160˚C. P3HB4HB polymers show high tensile strength and higher elongation at break. PHB has been blended with poly(vinyl acetate) (PVAc), two polymers being miscible, achieving good properties and high biodegradability of the products.[3] There are several different types of commercially produced PHA polymers, from random copolymers such as poly(3HP-co-4HB), poly(3HB-co-3HP), poly(3HB-co-3HV) (PHBV), poly(3HB-co-4HB) (P3HB4HB), to poly(3HB-co-3HHx) (PHBHHx). Mixed type scl-/mclPHAs, such as P(3HB-co-mcl-3HA), are produced with the NODEX trademark. Production of poly 3-hydroxybutyrate-co-3-hydroxyhexanoate (PHBHHs) has been achieved through the PhaPCJ synthase in *Aeromonas hydrophila* cultures. Copolymers of P(3HHx-co-3HO-co-3HD-co-3HDD), showing a soft consistence, are also produced. Mixed polyesters containing poly-lactate (pLA), such as poly(LA-co-3HB-co-3HP) are produced industrially.[12,13]

PhaC: the PHA synthase

The PHA syntheses, PhaC, have been classified in four types, depending on the substrate used, and the tendency to produce short chain length (scl-) or medium chain length (mcl-) polymers: type I, type III, and type IV PhaC enzymes synthesize principally scl-PHAs, while type II phaC produce mcl-PHAs.[14] PhaC enzymes possess the catalytic triad C-H-D, cysteine, histidine, aspartate, similarly to lipases that prefer serine (S-H-D), and in proteases. The negatively charged aspartate, located at position 447 in $PhaC_{Cs}$ from *Chromobacterium* spp.[15] and position 480 in $PhaC_{Cn}$ from *Cupriavidus necator*, enhance the basicity of histidine, through a hydrogen bond: aspartate acts as a general base catalyst, accelerating de protonation of the 3-hydroxyl group in the alkane substrate. The PhaC enzymes differ in the structure of the PhaC, in type I and type II being formed by a monomer that homodimerize, while in type III and IV the catalytic subunit associates to another subunit, forming hetreodimers.

Type I and type II PHA syntheses

Type I and II PHA syntheses are formed by a single protein, about 60 kDa in size. The active PhaC enzyme is a dimer, with catalytic (CAT) domains facing each other, with N-terminal domains making direct contacts, supporting monomer interactions. It was suggested that a partially folded catalytic domain is occupied by a stretch of amino acids (LID) of the CAT domain, able to change conformation and to slide away in presence of activation factors, thus opening the catalytic domain, that become occupied by the 3HB-CoA substrate.[14] $PhaC_{Cn}$, a type I enzyme in *Cupriavidus necator*, is composed of 191 amino acids N-terminal domain, and a C-domain containing the catalytic site, composed of 398 amino acids, for a total of 589 residues (Table 1). In $PhaC_{Cn}$ the catalytic triad Cys_{319}, Asp_{480} and His_{508}, is located between the beta 6 and alpha 3 turn, the beta 10 and alpha 7 turn, and at the end of the beta 11 strand, respectively. Between the Cys_{319} and the Asp_{480} there are the Dimerization loop (D-loop) and the helix-turn-helix motif (HTH). $PhaC_{Cs}$ from *Chromobacterium* strains has the property to use either 3HB, 3HV, and 3HH, as substrates, with production of scl-PHAs with mixed composition, and ability to incorporate 3OH-C5 and 3OH-C6 alkanes into the polymer. $PhaC_{Cs}$ has a fast polymerization rate, is composed of 560 amino acids (57 kDa in size) but, being shorter amino acid positions change, and compared to $PhaC_{Cn}$. $PhaC_{Cs}$ structure has the substrate-binding site hidden by a partially disordered protein domain, the CAP domain, that closes the active site access, with the Cys_{291} in the catalytic site accessible to substrate when the LID domain slides away.

$PhaC_{Cs}$ due to a change of names with use of Greek letters in the N-terminal secondary structure, there are only six ß-strands (ß8 to ß13) and four a-helices (α4 to α7), whose number is reduced in respect to $PhaC_{Cn}$ secondary structure. In $PhaC_{Cs}$ the catalytic triad is positioned after the β9 sheet, in respect to the β11 sheet in $PhaC_{Cn}$ and Asp_{447} is located at the ß8-α4 fold, formed by ß8 strand and α4 helix, while in $PhaC_{Cn}$ this is the β10-α7 fold. Chuah[16] showed by mutation of amino acids that in $PhaC_{Cs}$ Ala_{479} is a critical residue required for substrate specificity, with production of different copolymers, as P(3HB-*co*-3HHx). In $PhaC_{Cn}$ and in other type I enzymes this position corresponds to the conserved residue Ala_{517}. In the study by check, Ala_{479} is located within α5 helix, with the side chain protruding into a depression of the molecular surface formed by loops β4-α1, β9-α5 and α5-β10 of the core sub domain, and is partially covered by the loop of the LID region: mutation of the A_{479} resulted in weakening of interactions between the LID region and the core sub domain, and stabilization of the active form of the enzyme, with release of LID region from the catalytic site. Ala_{479} is supported by polar residues (Ser_{475} and Arg_{490}), so that mutating Ala_{479} with Ser or The supports hydrogen-bonding interactions with the polar residues, stabilizing the α5 helix with the His_{477}, part of the catalytic triad. The enzyme $PhaC_{Cc}$ from *Caulobacter crescentus* (*C. vibrioides*) accommodates 3-hydroxyalkanoates with various alkyl side-chain length. Also in $PhaC_{Cc}$, Ala_{479} substitution has been reported to increase $PhaC_{Cc}$ substrate preference for 3HHx. In *Delftia acidovorans* the PHA synthase is larger, possessing a 40 amino acid residues stretch that improves the specific activity of the enzyme. *Aeromonas* species, principally *A. caviae, A. hydrophila*, and *A. punctata*, have type I PhaC. These syntheses have mixed substrate specificity, that are exploited in the production of mixed type scl-mcl-PHAs marketedunder the "Nodex" trademark. Type II PHA syntheses are present and distributed in various bacteria: *Pseudomonas* species contain two *phaC* genes, of which *PhaC1* codify for the active enzyme under physiological conditions. PhaC2 in *P. oleovorans* has a higher affinity for 3-hydroxyhexanoate (3HH). PhaC1 and PhaC2 in *P. stutzeri* has been applied to produce mcl-PHAs[16–19] in engineered bacteria. *Ps*PhaC2 with four mutated amino acids, $E_{130}D$, $S_{325}T$, $S_{477}G$, and $Q_{481}K$, was exploiteded to produce mcl-PHAs and block copolymers, being able to host in the active site substrates with various shapes and structures. PhaC syntheses have been described in *Halomonas* species. Tyr_{412} in $PhaC_{Cs}$, and Tyr_{446} in the α6 helix in $PhaC_{Cn}$, are residues conserved in type I, III and IV PHA syntheses, while Phe occupies this position in type II PHA syntheses. In addition, another amino acid showed to have a role in accommodating larger substrates, by means of mutagenesis studies. Tyr_{438} is conserved in type I, III and IV enzymes, while in type II PhaC this position is occupied by His: the elimination of the bulky side chain of phenol ring can reduce the size of substrate entrance of catalytic site, and change the polar interactions with other amino acids facing the substrate entrance tunnel.

Type III and IV PhaC syntheses

Type III PHA syntheses are composed of two subunits, the catalytic subunit, PhaC (40–53kDa) and a second subunit, PhaE (ranging from 20 to 40kDa), involved in formation of the PhaEC complex, in which PhaE is required for PHA polymerization. Type III PHA syntheses are tetramers, such as in phaEC from *Chromatium vinosum*. The results of enzyme analysis, referring to $PhaC_{Cn}$ and PhaC type III from *Chromatium vinosum*, describe the presence of the active site in which cysteine is buried in a pocket: Check and coauthors, compared the

crystal structure with other enzymes with other enzymes with resolved crystal structure (lipases), and proposed the substrate entrance and product exit channels. An enzyme belonging to type III phaC has been characterized in *Thiocapsa pfennigii*. There is an Archaeal type, type IIIA PhaC, represented by *Haloarcula marismortui*. Archea offer promising perspectives of application in bioplastics production, due to the easiness of PHB extraction. Type IV PHA syntheses from *Bacillus* spp. are composed of the catalytic subunit, PhaC (41.5kDa) and a second subunit, PhaR (22kDa. Type IV PHA syntheses are classified as *Bacillus cereus* type (IV$_c$), *B. megaterium* type (IV$_m$), and *B. bataviensis* type (IV$_b$). In recombinant *E. coli* expressing PhaRC from *B. cereus* YB-4, PHA is processed by the synthase alcoholysis and cleavage at the C-terminal. This reaction is exploited to regulate PHA molecular weight and to modify the PHA carboxyl terminal.[20]

Table 1 Bacterial species possessing PhaC synthases of different types, and species of PHA produced

Species	Type of PhaC	Catalytic triad: the lipase phaC box serves to form intermediate product Cys-S-H3B	MW, nr. of amino acids	Type of polymer	Mechanism
Cupriavidus necator (*Ralstonia eutropha*)	Type I (PhaC$_{Cn}$ / RePhaC1)	Cys$_{319}$, Asp$_{480}$, His$_{508}$	60 kDa, 589 residues	Short-chain-length homopolymers (scl-PHA, as PHB)	helix-loop-helix motif (HTH) and Dimerization loop support dimer formation
Chromobacterium spp., strain USM2	Type I (PhaC$_{Cs}$) with mixed substrate range, utilizes 3HB, 3HV and 3HH	Cys$_{291}$, Asp$_{447}$, His$_{477}$	57 kDa, 560 aa,	scl-PHA polymers with mixed composition	CAP and LID domain for opening the access and dimerization
Delftia acidovorans (*Comamonas acidovorans*)	Type I		61 kDa, 599 aa		
Aeromonas spp. (*A. caviae, A. hydrophila, A. punctata*, etc.)	Type I, with mixed substrate range			"Nodex", mixed-type scl-mcl-PHAs	
Caulobacter crescentus (*C. vibrioides*)	Type I, PhaC$_{Cc}$ accommodating 3-hydroxyalkanoates with various alkyl side-chain length		57 kDa, 560 aa	mixed-type scl-PHA	
Pseudomonas spp. (*P. putida, P. mendocina, P. oleovorans, P. campisalis, P. stutzeri*)	Type II, PhaC1, PhaC2 with high affinity for 3HH	Cys$_{296}$, Asp$_{452}$, His$_{453}$ and His$_{480}$ (it has been proposed that the first His. His$_{453}$ is the catalytic histidine)		synthesis of mcl-PHA	
Halomonas spp., (*H. campisalis, Halomonas* sp. O-1, *H. elongata* DSM2581	Type II, polymerizing mcl-PHAs			mcl-PHAs	
Chromatium vinosum	Type III			Scl-PHA	active site cysteine buried in a pocket forming substrate entrance (3OH-alkanoyl-S-CoA), and product exit channel
Thiocapsa pfennigii	Type III, hybrid Pha synthase			Scl-PHA	easiness of PHB extraction
Haloarcula marismortui	Type IIIA (archeal)			Scl-PHA	
Bacillus cereus	Type IV	C$_{151}$, Asp$_{306}$, His$_{335}$	41 kDa	Scl-PHA	Alcohol lysis is shared among type IV PHA syntheses: regulation of PHA molec. weight and modification of the PHA carboxyl terminus
B. bataviensis	Type IV		41 kDa	Scl-PHA	
B. megaterium	Type IV	C$_{147}$, Asp$_{302}$, His$_{331}$	41 kDa	Scl-PHA	

PHA production in biofermentors

The methods for PHA production in fermentors are industrially important: most production plants make use of patented strains, engineered PHA syntheses, and growth conditions favoring cell high yield and high PHA content/dry cell weight. Recent reviews have described most of the updated processes and applications of bacteria in bioplastics production.[21] Bacterial cultures require sterilization; therefore some author proposed the use of halophiles have been proposed to reduce costs of industrial production. Extraction of PHAs from bacteria requires costly processes and methods, that have been described in reviews.[1,22,23] Bacteria have been modified to sustain granule formation under high pressure, capable of autolysis in determined conditions.[24,25] Researchers proposed the use of Archaea or cyanobacteria that have PHA granules easy to be extracted, to decrease the costs of production.

Biosensors for automatic monitoring of fermentation process

In the monitoring PHA synthesis in biofermentors, various methods have been described, from PHA/lipid staining[26] and analysis of fluorescence intensity, to flow cytometry,[27] to physicochemical analyses (Raman spectrometry, FTIR, SERS).[28] Various detection systems have been applied to quantify the bacteria, with optimum condition for PHA extraction at OD=25-50: the proposed analyses well suited for bacteria concentration evaluation range from spectrophotometric detection, as Fourier Transformation-IR spectrometry (FTIR) and Reflectometric Interference Spectroscopy (RIfS), to Surface Enhanced Raman Spectroscopy (SERS), to electrochemical biosensors, such as Alternate Current (AC) susceptometry. In industrial fermentation for production of PHA, several devices have been proposed to monitor the variables of the process. In particular, the determination of bacteria biomass, the quantification of carbohydrates in the feedstock, and the measure of PHA produced. These control of these parameters can optimize the use of bioreactor for the period of use (reaching the desired bacterial biomass in the shortest time), maximizing the produced PHA at shortest operating times. Various sensors have been applied to biofermentors in PHB production.[29] This is a critical point in fermentation, since bacteria that do not synthesise PHAs still consume sugars: often sugars are completely consumed during exponential phase growth so that new feedstock supply is necessary. Traditional PHA screening methods for PHA quantification in whole cells have been based on Nile Blue staining and fluorescence detection.[30] Nile Blue dye stains PHB and other neutral lipids, therefore a medium rich in lipids, such as vegetable oils, is easily stained a specifically. New methods have been proposed, such as staining of PHA with lipophilic fluorescent LipidGreen1, and may be run in automatic, providing measures even over-night.[26] A quantitative measure of PHA producing bacteria has been based on fluorescence scale reads using a laser scanner.[31] (Figure 1) In our hands, the system showed an optimization of feed stock consumption and determination of feed supplementation during exponential growth that occurred contemporary to PHB synthesis, while at the stationary phase determination of maximum PHB presence allowed to stop the biofermentor without further supplementation of feeds. In this way, we obtained an improvement of bacteria growth phase monitoring, in respect to the available systems. Even at low bacterial concentrations, as shown by Optical Density of 2.4 in Figure 1, growth was maximized through evaluation of nutrients requirement, while no nutrient was added when PHB synthesis reached its peak, with containment of materials and costs of biofermentor maintenance. At higher O.D. (i.e. 25 or higher), the system could allow to obtain a high yield of PHB/dry cell content.
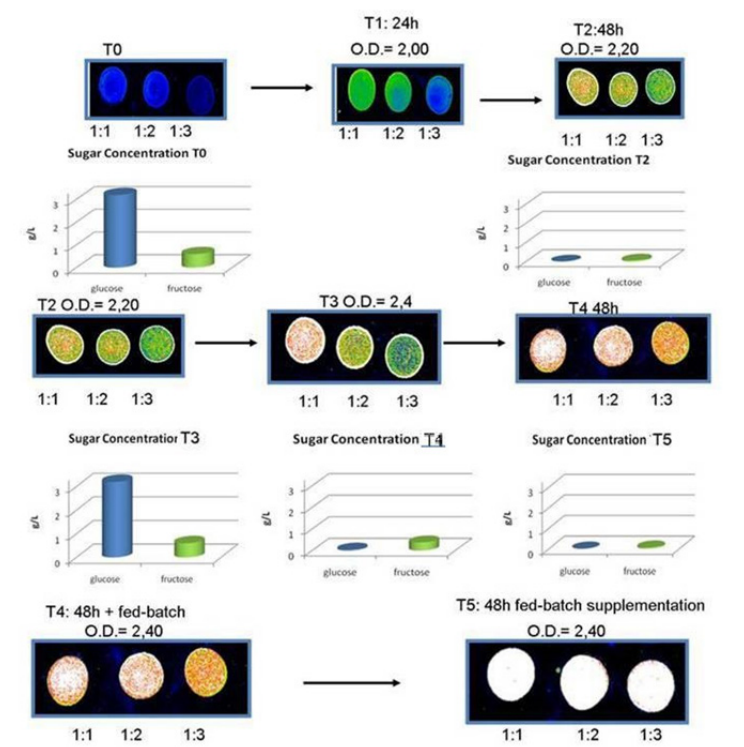

Figure 1 48 hour fermentation in biofermentor ad monitoring of PHB synthesis using Nile Blue staining of bacterial cells and sugars consumption using enzymatic kit. Bacteria were used as total suspensions or diluted 1:2 and 1:3 that were spotted onto glass slides and analyzed on an Affymetrix 428 array Scanner, at excitation/emission 460 nm/550nm. The dilutions showed increase in fluorescence until saturation of signal (white spots). T2 required addition of feed stock, until sugars were totally consumed (T4 and T5).

To the aim of automated detection of PHA in bacteria in fermentors, SERS methods could be effectively applied not only to quantify PHAs, but also to discriminate the type of PHAs produced. This may help to analyze the quality and quantity of PHAs at the same time. The combination of various sensors could make easier the use of bioreactors at industrial scale, optimizing the time of use (bacterial growth) and maximizing the synthesis of PHAs in the shortest time possible. Among the measuring methods or applied to biofermetors, the most favoured are biosensors with few equipment maintenance and less technical involvement. EIS, SPR and other applications require calibration curves, while optical measurements require minimal liquid handling, such as dilution steps and sample replicas. Biofermentors work with high concentrations of sugars in the medium. In the quantification of sugars consumed, and analysis of feedstock, several methods are present based on sticks or test strips, using viscosimetric[32] or amperometric detection of glucose oxidase activity. Nano encapsulation of enzymes to read glucose concentration offer also an opportunity for biosensing.[33]

PHA quantification and extraction

Several approaches have been proposed to make the process economically convenient. For instance, single cell protein method allows fermentation and recovery of granules in a single passage;[34–36] there are various lysis methods available,[26,37] based on alkaline treatment, before PHA recovery.[38] All these protocols are economically convenient and environmentally friendly, not requiring the use of solvents such as chloroform. To measure intracellular polymers, direct cellular digestion in sulphuric acid has been applied. For instance, 50μL of medium are added into 500μL of 96% sulphuric acid in a water-bath at 95-98˚C until PHA is converted to crotonic acid. Spectrophotometric analysis at 235nm is then performed using a quartz cuvette. The PHA concentration is defined as gram of polymer/litre of culture. To extract PHA polymers, cell lysis is obtained by enzymatic digestion. After lysozyme digestion for 1h, a digestion with proteinase K is performed. Then, the digested cell material is transferred in a glass tube. Hot chloroform is added and samples are kept in a water-bath at boiling temperature for 2h (vortexing every 10min). Centrifugation at 3000g for 20minutes allows to remove non-PHB cell material and to recover the lower chloroform phase, containing PHB polymers. Finally, solid PHB/PHA is obtained by non-solvent (methanol and water 7:3vol/vol) precipitation (five volumes for each volume of chloroform) followed by filtration.[31]

Conclusion

In the near future an increasing interest in industrial production of PHAs with different composition is envisaged. This will attract attention to modified PHA syntheses, engineering biosynthesis pathways, and optimization of fermentation conditions to produce bioplastics at convenient costs.

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Conflict of interest

Authors declare that there is no conflict of interest.

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