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ORIGINAL PAPER

Make it or break it: A review on PHA synthase and depolymerase proteins

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Abstract
Petroleum-based plastics are recalcitrant world-wide used materials that severely pollute the environment, thus biodegradable bioplastics are emerging as a viable alternative. From this group, the study of polyhydroxyalkanoates (PHAs) has stood out for their potential in diverse applications including medicine, packaging and agriculture. The enzyme responsible for PHAs synthesis inside the microbial cell is the PHA synthase (PhaC). PhaCs are present in a wide variety of microorganisms and are classified according to their substrate specificity and subunit composition into 4 classes. Class I, class III and class IV use the acyl-CoA as a precursor to synthesize short-chain-length PHAs while Class II enzymes use an intermediate of the β-oxidation pathways to synthesize medium-chain-length PHAs. Enzymes from this pathway that have been upregulated and downregulated to optimize PHAs production are described in this review. Another important enzyme is the PHA depolymerase (PhaZ) which is responsible for all PHA degradation inside and outside the cell. This review describes both enzymes in detail, including classification, structure, substrate specificity and proven protein engineering techniques for enzymatic rate enhancement and modified substrate specificity of the proteins. It also includes a mutation map for the class II PhaC sequence of *Pseudomonas putida* that suggest point mutations for future protein engineering work.

Keywords Polyhydroxyalkanoates (PHAs) · Bioplastics · PHA synthase (PhaC) · PHA depolymerase (Z) · Plastic pollution · Protein engineering

Introduction

Polyhydroxyalkanoates (PHAs) are biopolymers classified as polyesters which many bacteria and archaea accumulate in the form of intracellular granules as a reservoir of energy and carbon in response to stress conditions, especially when nitrogen is limited [1–4]. Maurice Lemoigne was the first scientist to isolate and characterize poly-3-hydroxybutyrate (P3HB), the simplest type of PHA, as a storage compound from *Bacillus megaterium* in 1926 [2, 5]. P3HB is a polymer composed of 4 carbon-chain-length repeating monomers, and it is the most common and abundant PHA in bacteria [6, 7]. PHAs are a good source of chemicals, polymers and biofuels [8].

Since this discovery, almost 100 years ago, thousands of papers have been published and some good reviews have been written describing the generalities of PHAs [9], their structure and composition diversity [10], applications in several fields such as: medicine [11], agriculture, biofuels and packaging [12]. There are also reviews describing potential suitable hosts for industrial production (such as *Cupriavidus necator*, *Pseudomonas*, recombinant *Escherichia coli* and yeasts) and their metabolic pathways [13].

This review re-capitulates some of the information mentioned in these reviews [14, 15], for example, main advantages and disadvantages of producing PHAs and the main metabolic pathway (β-oxidation pathway) is shown again [16] and expands to the specific genes that have been knockout and/or overexpressed from this pathway to enhance PHAs accumulation. It also compiles different methods for protein engineering and enzymatic evaluation as well

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as classification, structure and substrate specificity of both PhaC and PhaZ enzymes.

The PHA depolymerase (PhaZ) enzyme is responsible of depolymerizing the bioplastic and can be a useful tool for bioremediation of PHAs. Although, there are a volume of studies on PhaZ, for example the PHA Depolymerase Engineering Database published by Knoll, et al. [17], this volume is by far less than the volume of papers published on PhaC and review papers are greatly lacking. The reason PhaZ has been less studied than PhaC is because there isn't sufficient need for PHAs bioremediation as PHAs accumulation in the environment is still not a problem [18].

Another novelty of this work is the construction of a mutation map for the amino acid sequence of the PHA synthase (PhaC) from *Pseudomonas putida*. The purpose of the map is to show several potential amino acid change mutations that exist already in nature and that could be used to change the specificity/activity of a target sequence so that polymers with different monomer compositions could be created. The map is also accompanied by a table of single amino acid mutations that have already been studied and their effect over the enzyme.

Bioplastics

Bioplastics are a growing class of materials that can be used as an alternative to petroleum-based plastic [19]. All bioplastics are produced by living organisms from renewable feedstock. However, not all bioplastics are biodegradable [19]. The main types of bioplastics are illustrated in Fig. 1. Bioplastics have been studied for more than a century and yet, their industrialization is still embryonic [20]. According to the report of the European Bioplastics, bioplastics currently (2023) represent roughly 0.5% percent of the over 400 million tonnes of plastic produced annually [21].

The main advantage of bioplastics is that they have a faster biodegradation rate than petroleum-based plastics in a wide range of environments. Common petroleum-based plastics such as polyethylene, (PE) polystyrene (PS) and polyethylene terephthalate (PET) have a lifespan of 10–600, 50–80 and up to 450 years depending on the conditions [40, 41] while a PHA bottle is estimated to degrade in only 1.5–3.5 years in the marine environment [42]. In soil, with the appropriate temperature and moisture levels, PHA can degrade in as little as 7 weeks [43]. PHAs are also inexhaustible type of material which is an advantage over petroleum given that petroleum reserves are expected to run out by the year 2070 [44].

On the other hand, one of the main roadblocks to produce bioplastics is their higher costs. It has been calculated

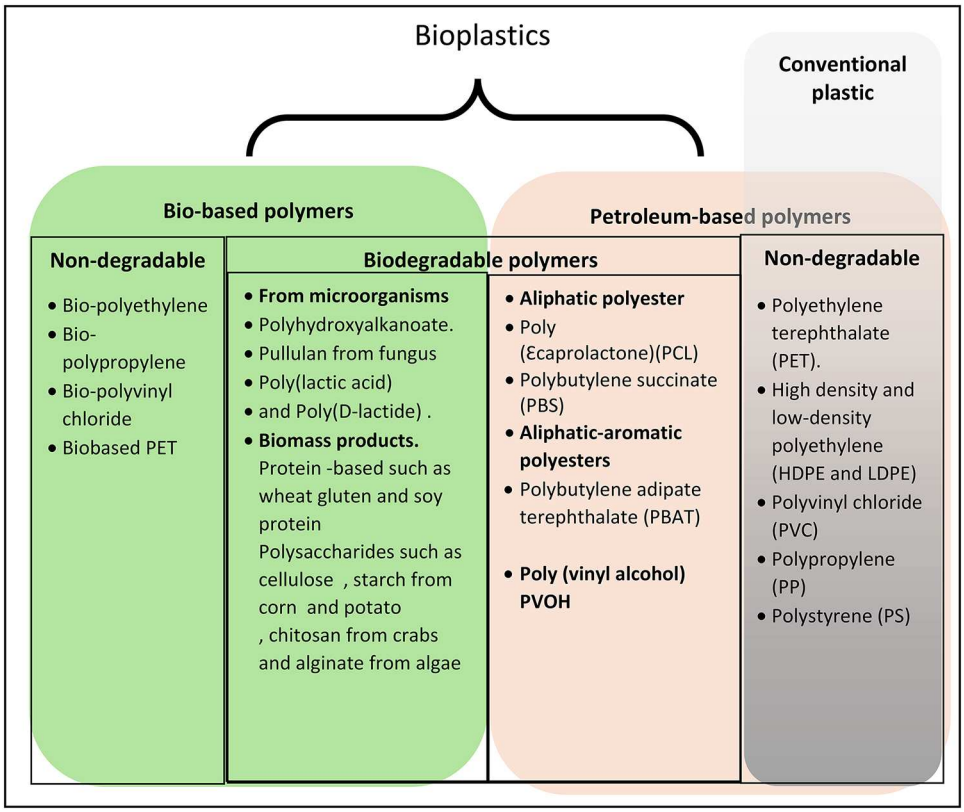


Fig. 1 Types of bioplastics modified from [22–39]

that the minimum selling price of PHAs in 2022 was 4.83 $/kg [45] while the global cost of, for example, high density polyethylene (HDPE) and of polyethylene terephthalate (PET) is ≈1 $/kg [46]. The high costs of PHA production comes mainly from the carbon feedstock used for the fermentation (≈38% of the production costs) [47]. Common industrial feedstocks include sugar beet, sugar cane bioproduct, canola oil, corn or cassava and sucrose [48–55]. Other high costs come from the fermentation itself and from downstream processing [56].

Additional disadvantages include the use of land to grow these feedstocks, and thermophysical properties of some types of these plastics such as thermal instability, brittleness, low melt strength etcetera [57].

Classification thermophysical characteristics and advantages of PHAs

PHA polymers are classified by their monomer length as short chain length (scl), which normally contain 5 or less than 5 carbons (≤C5), for example poly-3-hydroxyvalerate (P3HV) (P3HB falls in this category as well) and as medium-chain-length (mcl) which contain in between 6 and 14 carbons (≥C6, ≤C14), for example: poly-3-hydroxyoctanoate (P3HO) and poly-3-hydroxyhexanoate (P3HHX) [58–60]. Long chain length polyhydroxyalkanoates (lcl) contain more than 14 carbons and are a very rare type of PHA [61]. PHA polymers are composed of a backbone and a lateral chain (R) which is normally an alkane chain (Refer Fig. 2) [15].

Aside from alkanes, PHAs can have lateral chains containing double or triple bonds and /or include different functional groups such as halogen atoms, methoxy, benzoyl, ethoxy, cyanophenoxy, acetoxy, phenoxy, hydroxyl, nitrophenyl, epoxy, carbonyl, cyano and others [63].

There are PHAs in which the length of the backbone is longer, so the hydroxyl group to be esterified is not in carbon C3. Other PHAs have a thioester group in the place of the oxoester linkage. Some of these PHAs are uncommon

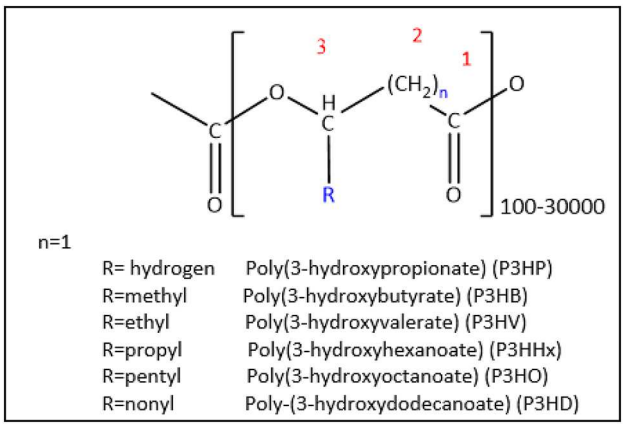


Fig. 2 *PHAs general formula* [15]. *Structure modelled using ChemDraw* [62]

and can only be obtained by chemical or physical modification of naturally occurring ones [7, 63, 64].

PHAs can also be classified as homopolymers and copolymers, for example P(3HB-*co*−3HV) is a copolymer made up from 3-hydroxybutyrate and 3-hydroxyvalerate monomers. The monomers are usually incorporated randomly across the polymer, thus they are also referred as random co-polymers [65]. This process depends on the carbon substrate, metabolic pathways available and on the specificity of the enzymes involved [66]. *Cupriavidus* and other organisms with PhaC type I use acyl-CoA from the metabolism of sugars to polymerize scl-PHA [14, 67–70] through the 3 enzymes from the operon phaCAB [66, 71] while organisms with PhaC type II, type III and type IV [14, 67–70] use the intermediate 3-hydroxyacyl-CoA from the metabolism of fatty acids (β-oxidation pathway) to produce mcl-PHA [66, 71].

Nevertheless, It is also important to mention that, while the natural substrate of PhaC in *C. necator* is 3HB-CoA, it was able to accept 3-hydroxyoctanoate-CoA and 3-hydroxydodecanoate-CoA to produce medium-chain-length PHA when expressed in *E. coli* suggesting that the substrate specificity of the different types of PhaCs is less strict than originally thought [72]. This leads us to believe that the type of PHA (scl or mcl) is not only dependent on the substrate specificity of the PhaC but also on the specificity of the other enzymes involved and the metabolic pathway used.

Carbon feedstock is also a major factor defining the polymer composition. For example, *Klebsiella* spp. can produce P(3HB-*co*−3HV) with a higher portion of 3HV using soy waste and with a lower portion of 3HV using malt wastes as carbon feedstock, while *Alcaligenes latus* and *Staphylococcus* spp. can only produce homopolymers of hydroxybutyrate using these same substrates [73]. In contrast, *Cupriavidus necator* (*Alcaligenes eutrophus)* is capable of increasing the proportion of HV inside P(3HB-*co*−3HV) when NH_4OH is present in the medium [74]. Commercial copolymers include P(3HB-*co*−4HB) produced using *Cupriavidus necator* by Metabolix, USA, P(3HB-*co*−3HHx) produced also using *C. necator* by Kaneka, Japan and using *Aeromonas hydrophila* by P&G, China and by Shandong, Lukang [55].

In addition to the above classifications, PHAs can be classified as block copolymers. Block copolymers are macromolecules composed of sequences, or blocks, of chemically distinct repeat units. Hence, PHAs block copolymers are composed of different sets of PHA homopolymers linked together. Possible block copolymer structures include A-B diblock, A-B-A, A-B-C triblock, or (AB)n repeating multiblock [75]. It has been demonstrated that alternating between different carbon feedstocks leads to the formation of block copolymers; For instance *Cupriavidus necator* can synthetize poly(3-hydroxybutyrate)-

block-poly(3-hydroxybutyrate-*co*−3-hydroxyvalerate) P(P3HB-b-P3HBV) when valeric acid containing substrate is alternated with non-valeric acid substrate [76].

This changes on monomer composition affect the overall characteristics of the polymer. Comparison of the thermophysical characteristics between short-chain-length and medium-chain-length polyhydroxyalkanoates are illustrated in Table 1.

PHAs, in general, can be used in medical devices such as suture threads, patches, meshes, implants, tissue engineering scaffolds and controlled drug delivery systems. In particular, due to their elasticity, medium-chain- length PHAs have been reported to be used for soft tissue implants [80]. Moreover, there is evidence showing that the cytotoxicity of polyhydroxyalkanoates is inversely proportional with the length of the side chain of the monomer (longer side chains are less cytotoxic) making medium-chain- length PHAs better candidates for medical applications [81].

Mcl-PHAs are specially utilized for cardiac tissue engineering applications and heart valves because elastic materials are more suitable to be used as leaflets inside the tri-leaflet valve [81]. They also mimic better the mechanical properties of soft nerve tissue [82]. Scaffolds of the co-polymer P(3HB-*co*-3HHx) compared against scaffolds with scl-PHAs monomers showed stronger potential to promote differentiation of neural stem cells into neurons and therefore showed biomedical potential for repairing the central nervous system [83]. However, studies remain limited mainly to P3HO and the co-polymer P(3HB-*co*−3HHx) which are the only mcl-PHAs in large quantities [82].

One of the main advantages of PHAs is its high biodegradability compared to petroleum-based plastic. For example, a PHA-made water bottle would be expected to completely degrade in 1.5 to 3.5 years in marine environment [42] while a high-density polyethylene bottle has a marine half-life of 58 years [84]. However they also present some disadvantages such as the high cost of production [85]. Other advantages and disadvantages are listed below:

Advantages.

• Less CO_2 emissions and sustainability [18].

Table 1 Comparison of the thermophysical characteristics between short-chain-length and medium-chain-length PHAs

Short-chain-length PHAs	Medium-chain-length PHAs
Highly crystalline [77, 78]	Low crystallinity [77, 78]
Hard [77, 78]	Soft [77, 78]
Brittle [77, 78]	Flexible [77, 78]
	Thermo-elastomeric polyesters [77, 78]
	Low glass transition and melting temperature [77, 79]
	Low tensile strength and modulus [77, 79]
	Higher elongation at break [77, 79]

• The industrial production has a low safety risk compared to petroleum-based plastic production which includes flammable and toxic by-products [18].

• Waste water is non-toxic [18].

Disadvantages

• Not economically competitive compared to petroleum-based plastic [18].

• The monomer composition of the polymer is difficult to control [18].

• Difficulties in processing due to their low crystallization process [18].

• Heavy water consumption [18].

• Substrate to product conversion is low [18].

Metabolic pathways and metabolic engineering to optimize medium-chain-length (mcl) PHAs cell production

The monomer carbon chain length composition of mcl-PHAs is directly influenced by the carbon length of the fatty acid provided [86]. In each complete β-oxidation cycle, 2 carbons are removed from the fatty acid chain and cycles will continue to take place in the cell until the fatty acid is completely metabolized, as a result, monomers of different sizes will integrate into the PHA polymer to create a heteropolymer. The difficulty of controlling the polymer's monomer composition is one of the main challenges of mcl-PHAs scale up production. This difficulty is even greater in *Pseudomonas* because it has several gene copies for each of these β-oxidation pathway enzymes.

Nevertheless, weakening the β-oxidation pathway, by knocking out one or more copies of one gene, has proven to increase the carbon flux towards enoyl CoA (mcl-PHAs precursor) and avoid the degradation of the fatty acid substrate, which will enhance PHAs accumulation inside the cell [87, 88] and result in more homogenous PHAs in terms of monomer composition [88, 89].

The most common method to weaken the β-oxidation pathway is through gene knockout of *fadB* and *fadA* genes (See Fig. 3; Table 2). However, similar results have been achieved when using the β-oxidation pathway inhibitor acrylic acid (See Fig. 3; Table 2) [90]. On the other hand, mcl-PHAs accumulation can also be enhanced by overexpressing genes that are related to the β-oxidation pathway such as *phaJ*, *phaG*, *fabG* and *alKk* (See Fig. 3; Table 2) [91, 92]. No change in monomer composition is expected to take place nor has been observed when overexpressing these enzymes [91]. However, more research is needed to observe if substrate specificity of these enzymes can also play a role in monomer composition.

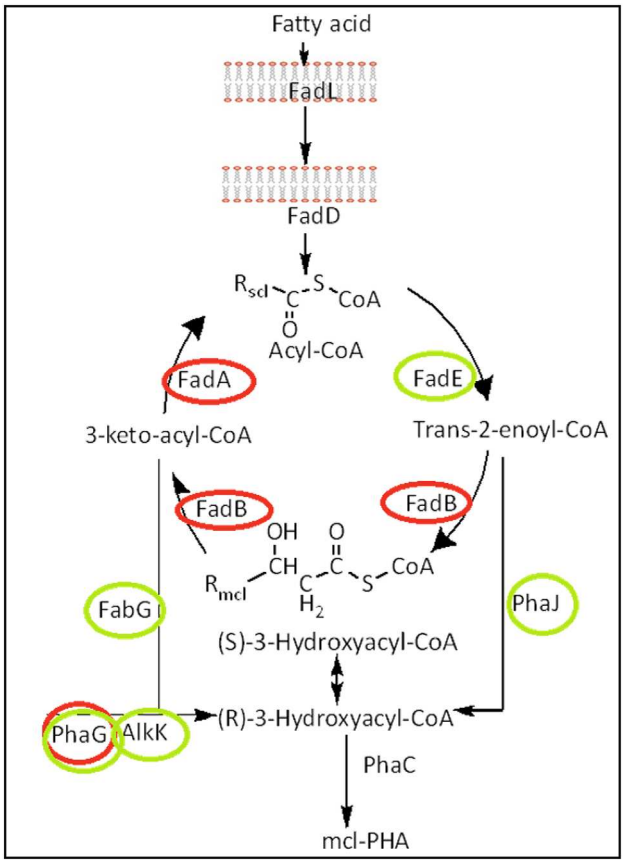


Fig. 3 Representation of the β-oxidation pathway and β-oxidation pathway-related genes. Genes circled in red have promoted PHAs accumulation when inactivated in several studies (Refer to Table 2). Genes circled in green have promoted PHAs accumulation when overexpressed (Refer to Table 2)

More than 5000 species of bacteria distributed in more than 14 bacterial groups including *Firmicutes*, *Bacilli*, *Clostridia*, *Actinobacteria*, *Micrococcal*, *Streptomycetales*, *Corynebacterial*, *Cyanobacteria/Melainabacteria* group, *Proteobacteria*, *Alpha proteobacteria*, *Gamma proteobacteria*, *Betaproteobacteria*, *Delta/Epsilon* subdivisions and other groups have the capacity of synthetizing PHA [66, 70]. Even some and halophilic archaea can accumulate it [106, 107].

However, for use in pilot or industrial scale production, reasonably high productivities of PHAs using fed-batch fermentation should exist in a range of (1 g L^{-1} h^{-1} to 2 g L^{-1} h^{-1}) for the process to be economically feasible according to Blunt, et al. [108]. The study of Jiang, et al. [90] using *P. putida* does fit this criteria and achieves a production of 1.8 g L^{-1} h^{-1} but most of the studies presented on the table above (Table 2) have a much lower productivity. For example, PHAs production in the study in which *Ralstonia eutropha* (Now *Cupriavidus necator*) accumulated 71% of its dry weight of the copolymer P(HB-*co*−17%-HHx) equivalates to a final ≈ 2 g/l after 72 h of cultivation [94] while in the study of Bhatia, et al. [96] using *Cupriavidus necator* as well fed with coffee waste achieved a productivity of approximately only 0.7 g/l in 72 h. This information leads us to believe that some of the studies that already exist are still not good candidates for scale up and industrial production and more optimization work is needed.

Additionally, there are other strains that have potential as industrial mcl-PHA producers and other genetical modifications, different than β-oxidation modifications, are efficient [109]. For example, genes for the consumption of xylose (*xylA*, *xylB* and *tktA*) were overexpressed along with the PhaC from *Aeromonas* inside the strain *Burkholderia sacchari* to produce 55.5% CDW of P(HB-*co*-HHx) [110].

PHA synthase (PhaC)

Classification

PHA synthases are divided into four classes based on their substrate specificity and on their subunit composition. Classes I, III and IV prefer to synthesize scl-PHAs while class II PhaC synthesize mcl-PHAs [14, 111]. (see Table 3) Yet, new studies show a volume of exceptions to this classification. For example, PhaC2 from *Pseudomonas stutzeri* 1317, (type II PHA synthase) can incorporate several short-chain-length and medium-chain-length monomers into PHAs [112]. *Chromobacterium* sp. USM2, can also utilize 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HHx) monomers [113] while *Aeromonas caviae* can synthesize the random co-polymer P(3HB-co-3HHx) [1].

Interestingly, the PhaC from *Rhodococcus pyridinivorans* BSRT1-1 can synthesize the scl-mcl co-polymer P(3HB-co-3HHx) when expressed recombinantly in a *C. necator* PHA negative strain [114]. The PhaC from the scl-PHA industrial strain *C. necator* can also broaden the range of monomers that is can take and incorporate mcl monomers into the polymer when expressed recombinantly in *E. coli.*

These important findings suggest that the PhaC specificity is not as strict as previously thought and that the monomer composition is not only dependent on the PhaC's substrate specificity but also on the specificity of the other enzymes from the pathway.

Structure and sequence

PHA synthases belong to the *α/β*-hydrolase superfamily and have only 8 strictly conserved amino acid residues. This so-called *α/β*-hydrolase fold consists of a characteristic succession of alpha helices and beta strands, usually found in

Table 2 mcl-PHAs accumulation enhanced by metabolic engineering of the β-oxidation pathway and the β-oxidation pathway-related enzymes

Organism used	B-oxidation pathway modification	PHAs yield	% of mcl-monomer	Substrate used	
Ralstonia eutropha	Heterologous expression of *phaC* from, *Rhodococcus aetherivorans* I24, Expression of *phaJ* from *P. aeruginosa* to increase PHAs accumulation, Modification of the *phaB* activity	71 wt% 66 wt%	P(HB-*co*−17%-HHx) P(HB-*co*−30%-HHx)	Palm oil	[93]
Cupriavidus necator (*Ralstonia eutropha*)	*phaC* from *R. aetherivorans* I24 and *phaJ* from *P. aeruginosa* expressed in plasmid pCB113	45 wt% 1.3 g/l PHAs	P(HB-*co*−70%HHx)	Crude palm kernel oil	[94]
Ralstonia eutropha	Introduction of crotonyl-CoA reductase from *Streptomyces cinnamonensis*, *phaC* and *phaJ* from *A. caviae*	48 wt% 1.48 g/l CDW	P(HB-*co*−1.5%HHx)	Fructose	[95]
Ralstonia eutropha	Overexpression of enoyl coenzyme-A hydratase (*phaJ*) and *phaC2*. Deletion of acetoacetyl Co-A reductases (*phaB1*, *phaB2* and *phaB3*)	69 wt%	P(78%HB-*co*−22%HHx)	Coffee waste oil	[96]
Pseudomonas putida KCTC1639	Overexpression of *phaJ* Overexpression of *fabG*	27wt% 0.51 g/l PHAs (FabG overexpression depressed PHAs production)	NI	Octanoic acid	[91]
Cupriavidus necator	Expression of *fadE* from *E. coli* and *phaJ1* from *P. putida* KT2440, in plasmid pMPJAS03	46.1wt%, 4.1 g/l CDW 38.3 wt%, 3.28 g/l CDW	P(99%HB-*co*−0.37%HV-*co*−0.27%HHx-*co*−0.21%HO-*co*−0.08%HD) P(99.39%HB-*co*−0.33%HV-*co*−0.18%HHx-*co*−0.10%HO)	Canola oil Avocado oil	[92]

Table 2 (continued)

Organism used	B-oxidation pathway modification	PHAs yield	% of mcl-monomer	Substrate used	
DH5α *Escherichia coli*	Deletion of key genes in β-oxidation pathway and overexpression of acyl-ACP thioesterase (BTE), *phaJ3* and *phaC2* from *P. aeruginosa* PAO1 and *PP_0763* from *P. putida* KT2440 strain (*fadRABIJ*)	0.93 g/l CDW 0.75 wt%	P(100%HDD)	Decanoic acid	[97]
Escherichia coli W3110 *Escherichia coli WA101*(*fadA* mutant)	Expression of *fabG* from *E. coli* and *phaC2* from *Pseudomonas* sp 61 − 3 Expression of *rhlG* from *P. aeruginosa* and PhaC2 from *Pseudomonas*. sp 61 − 3 Expression of *fabG* from *E. coli* and *phaC2* from *Pseudomonas*. sp 61 − 3	4.8 wt%, 1.73 g/l CDW, 0.08 g/l PHAs 3.2wt%, 1.20 g/l CDW, 0.04 g/l PHAs 22.1wt%, 0.98 g/l CDW, 0.22 g/l PHAs	P(11%HHx-*co*−39%HO-*co*−50%HD) P(47%HO-*co*−53%HD) P(7%HO-*co*−93%HD)	Sodium decanoate	[98]
Cupriavidus necator	5 different transformants harboring *phaC* BP-M-CPF4 gene. (*phaJ* from *P. aeruginosa* expression increased HHx proportion). (*phaB* and *phaA* genes expression modify composition)	(48.9–83.7) wt% (3.6–6.2) CDW (2.1–1.4)g/l PHAs	HHx(1–18%)	Palm olein Palm kernel oil	[99]
Pseudomonas putida KT2440	Deletion of *phaZ*, *fadBA1* and *fadBA2* and overexpression of *phaG*, *alkK*, *phaC1* and *phaC2* (strain AG2162)	1.758 g/l CDW 54 wt% 0.657 g/l CDW, 17.7 wt%	No information	P-coumaric acid Lignin(corn stover)	[100]

Table 2 (continued)

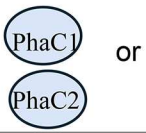
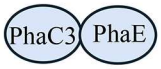
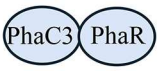
Organism used	B-oxidation pathway modification	PHAs yield	% of mcl-monomer	Substrate used	
Pseudomonas putida KT2440	No (acrylic acid inhibits β-oxidation pathway)	75.5 wt% 1.8 g/lh PHAs	P(89%HHp-*co*−11%HN)	nonanoic acid: glucose: acrylic acid (1.25:1:0.05)	[90]
P. putida KT2442	Deletion of: *fadB2x*, *fadAx*, *fadB*, *fadA*, 3-hydroxy-acyl-CoA dehydrogenase, acyl-CoA dehydrogenase and *phaG*.	9.19 wt% 1.03 g/l CDW	P(3HD-*co*−84%−3HDD)	Decanoic acid Dodecanoic acid	[88]
P. putida KT2442	Deletion of *fadB* and *fadA*, Deletion of *phaC* and replacement with *phaP-CJ*$_{AC}$ operon (KTOYO6ΔC (*phaPCJ*$_{AC}$) strain)	5.82 g/l CDW 57.80 wt%	58%PHB-block-42%PHHx	sodium butyrate, sodium hexanoate (1:2) alternating times	[101]
Pseudomonas entomophila LAC32	Weakening of β-oxidation pathway (Deletion of *fadA(x)*, *fadB(x)* and *phaG*) Insertion of *phaA* and *phaB* from *C. necator.* PhaC mutated from *P. putida* 61 − 3. Block of some genes in *de novo* fatty acid synthesis pathway	Not reported	different rations with mcl from 0 to 100%	Glucose and related fatty acids	[102]
Pseudomonas putida KT2442	*fadA* and *fadB* knockout mutant	84 wt%	P(41%HDD-*co*−59%HA)	Dodecanoate	[103]

Table 2 (continued)

Organism used	B-oxidation pathway modification	PHAs yield	% of mcl-monomer	Substrate used	
Pseudomonas mendocina NK-01	*fadA* and *fadB* knockout mutant (NKU-Δβ5) *fadA*, *fadB*, *phaG*, *phaZ* knockout mutant (NKU-Δ8) NKU-Δ8.	38 wt%, 1.7 g/l CDW 44 wt%, 2.3 g/l CDW 32 wt%, 1.3 g/l CDW	P(5.57%HHx-*co*−93.3%HO-*co*−1.05%HD) P(5.29%HO-*co*−94%HD) P(2.99%HO-*co*−28%HD-*co*−68%HDD)	Sodium octanoate Sodium decanoate Dodecanoic acid	[104]
Pseudomonas putida KT2442	Deletion of *fadA*, *fadB* (*P. putida* KTOY06)	2.86 g/l CDW 45.99 wt%	P(2.2%HHx-*co*−11%HO-*co*−21.6%HD-*co*−16.1%HDD-*co*−49%HTD)	Tetradecanoic acid	[105]

Scl: short-chain-length, mcl: medium-chain- length, PHA: poly(3-hydroxyalkanoate), PLA: polylactate, PGA: polyglycolate, PHB: poly(3-hydroxybutyrate), PHP: poly(3- hydroxypentanoate), PHHx: poly(3-hydroxyhexanoate), PHO: poly(3-hydroxyoctanoate),PHD: poly(3-hydroxydecanoate), PHDD: poly(3-hydroxydodecanoate).

Table 3 Polyester synthases can be divided into four classes

Class	Active state	Subunits	Species	Substrate preference	% identity from PhaC1(*C. necator*)
I	homodimer	60-73 kDa 	*Cupriavidus necator* *Chromobacterium* *Azohydromonas lata* *Aeromonas hydrophila*	Scl	100 46.88 61.64 40.41
II	monomer	60-65 kDa 	*Pseudomonas aeruginosa* *P. putida, P. oleovorans,* *P.spp, P. sp. 63-1*	Mcl	38.21 39.43
III	heterodimer	40 kDa 40kDa 	*Allochromatium vinosum*	Scl	26.44 – 66.67
IV	heterodimer	40kDa 20kDa 	*Bacillus megaterium* *Bacillus cereus.*	Scl	28.25 -50 27.67 - 50

[14, 67-70]. scl

lipases, where the catalytic residues aspartate, histidine and cysteine are. Out of the catalytic triad, the conserved cysteine residue is used as catalytic nucleophile [14, 115].

This cysteine is embedded in the PhaC box sequence (GS)-X-C-X-(GA)-G (X is an arbitrary amino acid), which is similar to the lipase box G-X-S-X-G proper of lipases, in the active site of the catalytic domain of the protein [115, 116]. In a posterior amino acid replacement study using PhaC from *Cupriavidus necator* the PhaC box sequence was proven to be still active when expanded to ([GAST]-X-C-X-[GASV]-[GA]). This study also shows the low mutational robustness of the last glycine residue as well as that of the central cysteine [111, 116].

In *C. necator*, the PhaC class I active enzyme is a homodimer with only one active catalytic site [115, 117]. Each monomer is composed of a single polypeptide chain of 65 kDa which contains an N-terminal domain of unknown function (residues 1–200) and a C-terminal catalytic domain where the polymerization takes place (residues 201–589) (Fig. 4, A**) (5T6O** Protein Data Bank elucidated using x-ray diffraction). The active site of each monomer is separated from the other by 33 Å across an extensive dimer interface.

The opening in the substrate access channel of the enzyme is near two arginine residues (one from each chain of the dimer), from which Arg_{398} is strictly conserved in class I PhaCs. This arginine along with the His_{481} are believed to be important for substrate HB-CoA binding and stabilizing while Arg_{421} may be involved in chain termination [115].

Figure 4B shows the structure of 3, class II, aligned PhaCs (PmePhaC, PpuPhaC and PstzPhaC) from *P. mendocina* NK-01, *P. putida* KT2440 and *P. stutzeri* 1317 respectively. The catalytic triad Cys, Asp and His are coloured

The catalytic domain of PHA synthase from *Cupriavidus necator* shares 29% sequence identity with the class III PhaC from *Allochromatium vinosum*, which suggests structural similarity with class III synthases. The catalytic domains of class II (*Pseudomonas*, Fig. 4B)) and IV PhaCs, despite of have being a lot less characterized, are also likely to be homologous, sharing around 40% and 30% identity with *C. necator* [115].

Substrate specificity and kinetics

Comparing K_m of different substrates gives some clues to the specificity of an enzyme. In the study of Zhang, et al. [117]. the much larger K_m value for 4HB-CoA compared with 3HB-CoA and 3HV-CoA indicates the importance of the distance between the hydroxyl group and the CoA moiety for substrate binding in the PhaC of *C. necator*, however, a longer side chain does not seem to strongly affect the substrate binding to PHA synthase of *C. necator*. (for

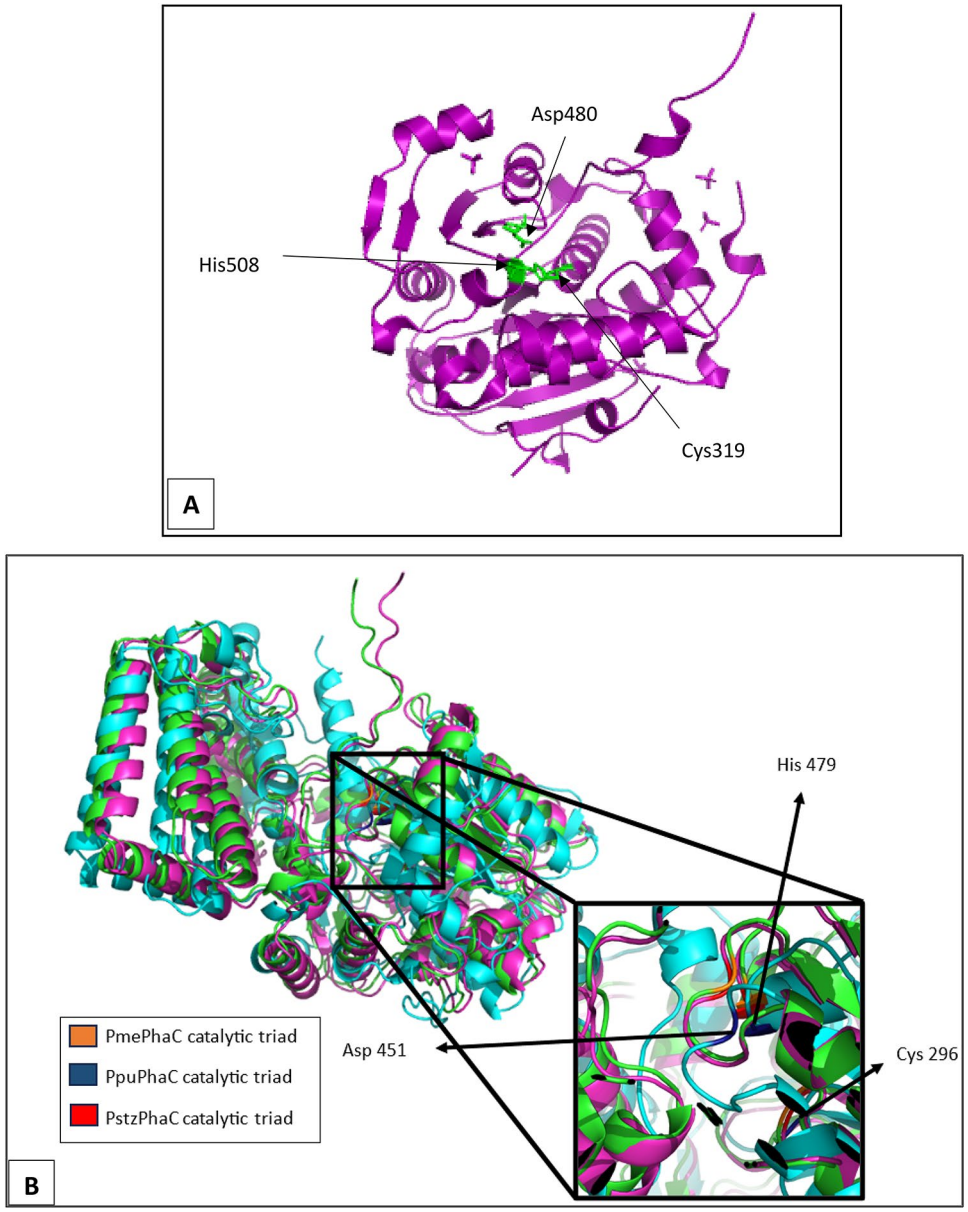


Fig. 4 (A) The catalytic domain of PHA synthase from C. necator. Catalytic triad is presented in green. [115] Molecule modelled in PyMOL for this work [118]. (5T6O Protein Data Bank elucidated using x-ray diffraction) (B) Pymol structure of 3 aligned PhaCs (PmePhaC, PpuPhaC and PstzPhaC) from P. mendocina NK-01, P. putida KT2440 and P. stutzeri 1317 respectively. The catalytic triad Cys, Asp and His are coloured.

comparison of more substrates and its K_m read Table II from Yuan, et al. [119].) It is also important to mention that, while the natural substrate of PhaC in *C. necator* is 3HB-CoA, it has been able to accept 3-hydroxyoctanoate-CoA and 3-hydroxydodecanoate-CoA to produce medium-chain-length PHAs when expressed in *E. coli* suggesting that it might have a broader substrate specificity than previously found [72].

Point mutations that might affect substrate specificity.

Proven PHA producing *Pseudomonas* strains include: *Pseudomonas mosselii* [120, 121], *Pseudomonas corrugata* [122–124], *Pseudomonas mediterranea* [122, 123], *Pseudomonas putida* [124–126], *Pseudomonas mendocina* [127, 128], *Pseudomonas chlororaphis* [129, 130], *Pseudomonas stutzeri* 1317 [86, 131],*Pseudomonas entomophila* [65, 102]., *Pseudomonas oleovorans* [132] and others.

For this literature review, an amino acid mutation map for the industrially PHA outstanding strain *P. putida* [133–135] was built following the following procedure: The amino acid sequence from the strain (NCBI: WP_010955566.1) was aligned against the NCBI database using the online tool BLAST [136]. The first 50 strains from the search were selected and using all the mutations observed, a mutation map was built and shown in Fig. 5 (Mutations shown in red). The occurrence for each mutation (how many times the amino acid was substituted in our pool of sequences) was also recorded and added on the SUPPLEMENTARY material.

Additionally, a literature search was conducted to identify point mutations that have already been tested in the laboratory. These mutations are shown in blue in Fig. 5 and described in Table 4.

Substrate specificity can rely in as much as one amino acid., the substitution of Leu484 in PhaC from *Pseudomonas putida* for valine shifts the substrate specificity from 8 C to 4 C. This amino acid (484 amino acid) is adjacent to the catalytic triad and it is conserved as leucine in class II synthases, as valine in class III and as valine or isoleucine in class I (*C. necator*) and is critical in determining substrate specificity [138]. On the other hand, amino acids Ser326 and Gln482 (also near the catalytic triad) in *Pseudomonas spp.* are conserved residues, that when substituted for other residues (thr326, cys326 and lys482, arg482 respectively) have a contrary effect and increase the enzyme preference for producing PHB (C4) over mcl-polymers [131]. The reason why different amino acids can change the enzyme properties in different ways is probably because every amino acid has different physicochemical properties such as

Fig. 5 Mutation map of Pseudomonas putida. The catalytic triad is squared in blue. **Lab tested** mutations are represented in **blue** while **blast search** mutations are written in **red**. *If more than one amino acid is possible in that position then the position is represented by the ++symbol*

Possible mutations					
10	20	30	40	50	60
MSNKNNDELQ	RQASENTLGL	NPVIGIRRKD	LLSSARTVLR	QAVRQPLHSA	KHVAHFGLEL
S D	D M	L	FT M	I	M
70	80	90	100	110	120
KNVLLGKSSL	APDSDDRRFN	DPAWSNNPLY	RRYLQTYLAW	RKELQDWVSS	SDLSPQDISR
D IF NI	+ EG S	R+		N HE IG+	N DT
130	140	150	160	170	180
GQFVINLMTE	AMAPTNTLSN	PAAVKRFFET	GGKSLLDGLS	NLAKDMVNNG	GMPSQVNMDA
AH D	A		A	+	E
190	200	210	220	230	240
FEVGKNLGTS	EGAVVYRNDV	LELIQYSPIT	EQVHARPLLV	VPPQINKFYV	FDLSPEKSLA
	+ F	+	E	T	RG
250	260	270	280	290	300
RFCLRSQQQT	FIISWRNPTK	AQREWGLSTY	IDALKEAVDA	VLSITGSKDL	NMLGACSGGI
Y E T+		H	V	+ D	
310	320	330	340	350	360
TCTALVGHYA	AIGENKVNAL	TLLVSVLDTT	MDNQVALFVD	EQTLEAAKRH	SYQAGVLEGS
	L R H	V +	L T	S	P G R
370	380	390	400	410	420
EMAKVFAWMR	PNDLIWNYWV	NNYLLGNEPP	VFDILFWNND	TTRLPAAFHG	DLIEMFKSNP
D		T	S		+
430	440	450	460	470	480
LTRPDALEVC	GTAIDLKQVK	CDIYSLAGTN	DHITPWPSCY	RSAHLFGGKI	EFVLSNSGHI
++ MK	P K +	T FCV A	QA	Q NV	+G
490	500	510	520	530	540
QSILNPPGNP	KARFMTGADR	PGDPVAWQEN	AIKHADSWWL	HWQSWLGERA	GALKKAPTRL
+GV	Y SKEM	A + L	S+ TN	Y + + CS	+ EP IV
550	559				
GNRTYAAGEA	SPGTYVHER				
SKA +V	A				

Table 4 Point mutations in Pseudomonas that affect substrate specificity

Source PhaC	Mutation	Host	Carbon source	Effect	
P. stutzeri 1317	Ser325Thr Gln481Lys	*Ralstonia eutropha* PHB^{-}4	Gluconate and Octanoate	Higher PHAs content and more affinity for PHB.	[113]
Pseudomonas 61 – 3	Ser325Cys Ser325Thr Glu481Lys Glu481Met Glu481Arg	*Ralstonia eutropha* PHB^{-}4	Fructose	Higher activity. The combination Ser325Cys with Glu481Met has higher activity but all combination worked. Mutation in position 481 produces higher molecular weight. (98–99% of short-chain-length)	[137]
P. putida GPo1	Leu484Val Ala547Val Gln481Met Ser482Gly	*P. putida* GPp104 PHAs^{-}	Sodium octanoate	Leu484Val increase PHB monomer content Ala547Val increase PHAs content. Gln481Met increases HHx monomer and PHAs content Ser482Gly increases HHx monomer and PHAs content.	[138]
Pseudomonas putida KT2440	Glu358Gly Asn398Ser	B-oxidation deficient *E. coli* LSBJ	Sodium dodecanoate	Higher PHDD production with higher molecular weight. Amino acids with hydrophobic and smaller residues either retained or increased PHDD.	[139]
Pseudomonas sp. 61 – 3	Ser325Cys ---TGC Ser325Thr—ACC Gln481Met Gln481Lys—AAG Gln481Arg—CGG	*E. coli*	Glucose	Double mutants increase PHB content. Codon picking is very important to enhance PHB content. **The mutation Ser325Thr, which exists in PhaC type I**, increases affinity for PHB.	[140]
Cupriavidus necator Aeromonas caviae	26% of the N-terminal of $PhaC_{AC}$ and 74% of the C-terminal of $PhaC_{Re}$	*E. coli* LSS218	Sodium dodecanoate	Using sodium dodecanoate, they obtained higher scl-mcl PHAs than the parental enzymes.	[141]
P. chlororaphis *P. sp.* 61 – 3 *P. putida* KT2440 *P. resinovorans* *P. aeruginosa* PAO1	Glu130Asp Ser325Thr Ser477Gly Gln481Lys	*E. coli* XL_1-Blue	Glucose Lactate	PhaC having mutations in these 4 sites were able to accept lactyl-CoA as substrate and produce PLA while wild types did not accumulate polymers.	[142]
P. sp. MBEL 6–19	Glu130Asp Ser325Thr Gln481Met	*E. coli* XL_1-Blue	Glucose	Increase the substrate specificity towards scl. Production pf P(HB-*co*-LA)	[143]
Pseudomonas stutzeri	Glu130Asp Ser325Thr Ser477Gly Gln481Lys	*E. coli*	Lactic acid	Terpolyesters (LA-*co*−3HB-3HP) Change of substrate specificity.	[144]
Pseudomonas sp.61 – 3	Gln508Leu Gln481Lys	*E. coli*		Mutation Gln508Leu is present in *C. necator*. Double mutation enhances PHB content.	[145]

hydrophobicity, charge, isoelectric point etc. In this regard Chuah, et al. [146] noticed that substitutions with uncharged residues resulted in enhanced PHA production.

Conservative mutations occur when an amino acid is substituted for another with similar physicochemical characteristics while non-conservative mutations occur when the amino acid change for another with completely different characteristics [147]. Non-conservative mutations are expected to have a bigger impact over the protein structure/function.

Another important characteristic of amino acids is their size and structure. In a study using the class I PhaC from *Chromobacterium* (short-chain length preference), the mutation A479S resulted in a 4-fold increase in 3HHx monomer incorporation and a 1.6-fold increase in PHA biosynthesis. Further analysis suggested that a change in size and the geometry of the substrate-binding pocket due to this mutation was determinant for PhaC yield and specificity [146].

Interestingly enough, the amino acid A479 is also present in PhaC from *Cupriavidus necator* H16 which is also a class I PhaC. This suggests that this mutation would most likely change the substrate specificity of this enzyme as well but experimental work is needed to confirm this hypothesis.

P**rotein engineering for catalytic enhancement**.

PHA synthase engineering is being performed for a variety of purposes that include improved PHAs accumulation yield, substrate specificity modification and/or higher molecular weight [148, 149].

The engineering strategies for these purposes include random mutagenesis, error-prone PCR mutagenesis, site-specific saturation mutagenesis, localized semi-random mutagenesis, gene shuffling, recombination of beneficial mutations and engineering of chimeric PHA synthases between others [149, 150]. Examples of enhanced PhaCs by enzyme evolution are shown in Table 5.

In addition to the above, PHA yield can be enhanced indirectly by modifying metabolic routes, for instance, the *β*-oxidation pathway to produce mcl-PHAs [66, 89], by recycling the CoA released from the transformation of 3-hydroxyacyl-CoA into PHAs (CoA inhibits PhaC) [160] or by the co-expression of molecular chaperones, which results in the synthesis of larger quantities of enzyme [161]. Another approach would be to use thermo-tolerant PHA synthases in order to enhance the bioconversion process and decrease the energy costs related with managing the exothermic fermentation process [149].

Methods to evaluate the enzymatic activity

The activity of PHA synthase can be determined by measuring the amount of CoA released from thioester-CoA during polymerization. The CoA in the medium is detected spectroscopically at 412 nm by reduction of 5,5-dithio-bis (2 nitrobenzoic acid) (DTNB), a compound which specifically reacts with thiol groups. One unit of enzyme activity is defined as the amount of enzyme that catalyses the release of 1 mol CoA/min. This technique, originally developed for short-chain-length PHA synthases was then modified for medium-chain-length synthases [162, 163]. It is the most common technique and it is quick to perform, unfortunately, hydrolysis reactions can lead to the release of CoA independently of polymerisation which can give false results [71].

Another way to measure PHA synthase activity is by measuring, spectrophotometrically at 236 nm, the hydrolysis of thioesters in substrates as described by Fukui, et al. [164]. In this technique one unit of enzyme activity is defined as the amount of enzyme necessary to convert 1 µmol of substrate to PHAs in one minute. It is a convenient technique but less accurate than measuring CoA release [71]. Later on, Gerngross, et al. [165] modified the technique and used a labelled substrate ([3-^{3}H]-hydroxyacyl-CoAs) [71, 165].

In the other hand, the enzyme activity essay developed by Kraak, et al. [132] is based on the analysis of substrate depletion by HPLC and production formation by gas chromatography and has proven to be highly accurate to measure the enzyme activity of medium chain length PHA synthases, but, the method is very time consuming.

The reliance on spectrophotometric assays has the drawback of hindering the simultaneous measurement of more than one enzyme activity [71]. Burns, et al. [166] developed a HPLC methodology to measure fluctuations in the concentration of CoA, acetyl-CoA, acetoacetyl-CoA, and β-hydroxybutyryl-CoA metabolites and to associate these fluctuations to the activity of an enzyme on the overall system.

A summary of the methods discussed in this section is shown in Table 6.

PHA depolymerase (PhaZ)

Classification

PHA depolymerases are carboxylesterases classified in 3 superfamilies according to the place where they are found in the cell (intracellular, extracellular, periplasmic) which at the same time are divided according to their substrate specificity (short or medium-chain-length) and according to their sequence similarities (presence of the lipase box and catalytic domain type).

Table 5 Studies of PhaC enhancement through synthetic PHA synthase evolution

Method	PhaC source	Changed enzyme property	Polyester	
Diversify PCR random mutagenesis	*Pseudomonas putida* KT2440	Higher yield with higher molecular weight	P3HDD	[139]
Error-prone PCR mutagenesis, saturation mutagenesis, in vitro recombination	*Pseudomonas* sp. 61 – 3	Higher yield	P3HB	[140]
Site-specific mutagenesis	*Pseudomonas* sp. SG4502	Substrate specificity	P(LA-*co*-3HB)	[151]
Localized semi-random mutagenesis	*Pseudomonas oleovorans* GPo1	Substrate specificity	P3HB, P3HA	[152]
Gene shuffling	*C. necator*	Higher yield, substrate specificity	P3HA	[153]
PCR-mediated random mutagenesis, intragenic suppression-mutagenesis	*Ralstonia eutropha*	Yield	P3HB	[154]
Chimeric PHA synthase	*P. oleovorans* *P. fluorescens* *P. aureofaciens*	Higher yield, substrate specificity	scl/mcl	[155]
Recombination of beneficial mutations	*Aeromonas caviae*	Molecular weight, fraction composition	P(3HB-*co*−3HHx)	[156]
Chimeric PHA synthase	*Aeromonas caviae* *C. necator*	Substrate specificity	P(3HB-*co*−3HHx-co-3HO)	[157]
Chimeric PHA synthase	*A. caviae* *C. necator*	Substrate specificity	3HHx / 3HB	[141]
Site specific mutagenesis	*Pseudomonas sp.* S64502	Substrate specificity	P(LA-*co*−3HB)	[151]
Site specific mutagenesis	*P. chlororaphis* P.sp.61 – 3 *P. putida* KT2440 *P. resinovorans* *P. aeruginosa* PAC1	Substrate specificity	P(3HB-*co*-LA)	[142]
Site specific mutagenesis Saturation mutagenesis	*Pseudomonas sp. MBEL 6–19*	Substrate specificity	P(3HB-*co*-LA)	[143]
Site specific mutagenesis, saturation mutagenesis	*Pseudomonas putida* GPo	Yield, substrate specificity	Copolymers with 3HB, 3HHx, 3HO, 3HD	[158]
None (natural mutation)	*Pseudomonas MBEL 6–19*	Substrate specificity	P(LA-*co*-GA-*co*−3HB) P(LA-*co*-GA-*co*−4HB), P(LA-*co*-GA-2HB)	[159]
Site specific mutagenesis	*Pseudomonas stutzeri*	Substrate specificity	P(LA-*co*−3HB-*co*−3HP)	[144]
PCR- mediated random mutagenesis Recombination of beneficial mutations.	*Pseudomonas sp.*61 – 3	Yield	P3HB	[145]

Scl: short-chain-length, mcl: medium-chain- length, P3HA: poly(3-hydroxyalkanoate),PLA: polylactate, PGA: polyglycolate, P3HB: poly(3-hydroxybutyrate), P3HP: poly(3- hydroxypentanoate), P3HHx: poly(3-hydroxyhexanoate), P3HO: poly(3-hydroxyoctanoate), P3HD: poly(3-hydroxydecanoate), P3HDD: poly(3- hydroxydodecanoate).

They can also be classified according to the type of PHAs they degrade which are, either native intracellular granules (amorphous)(nPHAs) or denatured extracellular granules (crystalline)(ds) as shown in Fig. 6 [6, 17].

Structure and sequence

PHA depolymerases have a catalytic triad (serine – histidine – aspartic acid) as active site. Similarly to PhaC, the catalytic serine is located in a G-X-S-X-G lipase box that is present in other α/β-hydrolases. Apart from this, a conserved non-catalytic histidine near the oxyanion hole, also present in lipases, can be found in PhaZ. It is important to note that there are also non-lipase box depolymerases [6, 17, 167].

The best studied PHA depolymerases are dPHAscl depolymerases which contain a short signal peptide, a catalytic domain (containing the oxyanion hole and the lipase

Table 6 Different methods to analyse PhaC activity

Method	Advantages	Disadvantages	Reference
CoA detection 412 nm	Quick	False positives due to other hydrolysis reactions that release CoA.	[71, 162, 163]
Thioesters hydrolysis detection 236 nm	Quick	Less accurate than CoA detection.	[71, 164, 165]
Substrate depletion and product formation analysis with HPLC and GC	Highly accurate	More time consuming	[132]
Co-A, acetyl-CoA and β-hydroxybutyryl-CoA analysis with HPLC	It is accurate and measures fluctuation in the overall system	Costly and time consuming	[166]

box), a short linker domain, and a substrate binding domain [17, 168]. In dPHAscl with catalytic domain 1 the oxyanion hole can be found in the N-terminal to the lipase box, like lipases, whereas in dPHAscl with catalytic domain 2 the oxyanion hole is found C-terminal to the catalytic triad. Example of microorganisms harbouring dPHAscl with catalytic domain 1 are *Sorangium cellulosum*, *Paracoccus denitrificans*, *Alcaligenes faecalis and Burkholderia mallei;* whereas examples *o*f microorganisms with catalytic domain type 2 are *Alteromonas macleodii*, *Pseudoalteromonas atlantica*, *Cupriavidus pinatubonensis* and others [6, 17].

Similarly, there are three types of short linker domain: fibronectin type (i.e. *Alcaligenes faecalis* [169]), cadherin-like domain (i.e. *Pseudomonas stutzeri* [170]) and 2 types of substrate binding domain (i.e. *Pseudomonas stutzeri* has both of these domains [170]) as shown in Fig. 7 [169, 171].

The dPHAscl catalytic domain Type II from *Penicillium funiculosum*, shown in Fig. 8 (**2D80** from Protein Data Bank elucidated using x-ray diffraction).is comprised of a single domain. The catalytic triad residues are Ser39, Asp121 and His155 and the amide groups of Ser40 and Cys250 form the oxyanion hole. A mutant revealed that Trp307 plays a role in the recognition of the ester group adjacent to the scissile group [172].

Crystallization of PHA depolymerase from *Paucimonas lemoignei*, whose catalytic triad special arrangement is similar to that one from *P. funiculosum* [173, 174] and from *Bacillus thuringiensis* [175] have also being achieved.

Substrate specificity and kinetics

PHA depolymerase catalyzes the reaction in Fig. 9.

PHA depolymerization occurs in two steps: adsorption by the substrate binding domain and then hydrolysis by the catalytic domain [176, 177]. The C-terminal amino acids of dPHAscl depolymerases represent the PHA-specific binding domain to the substrate (SBD) [168]. SBD has an interaction of 100 pN with the substrate [178] and such interaction consists of hydrogen bonds between hydrophilic residues in the enzyme and ester bonds in the polymer and between the

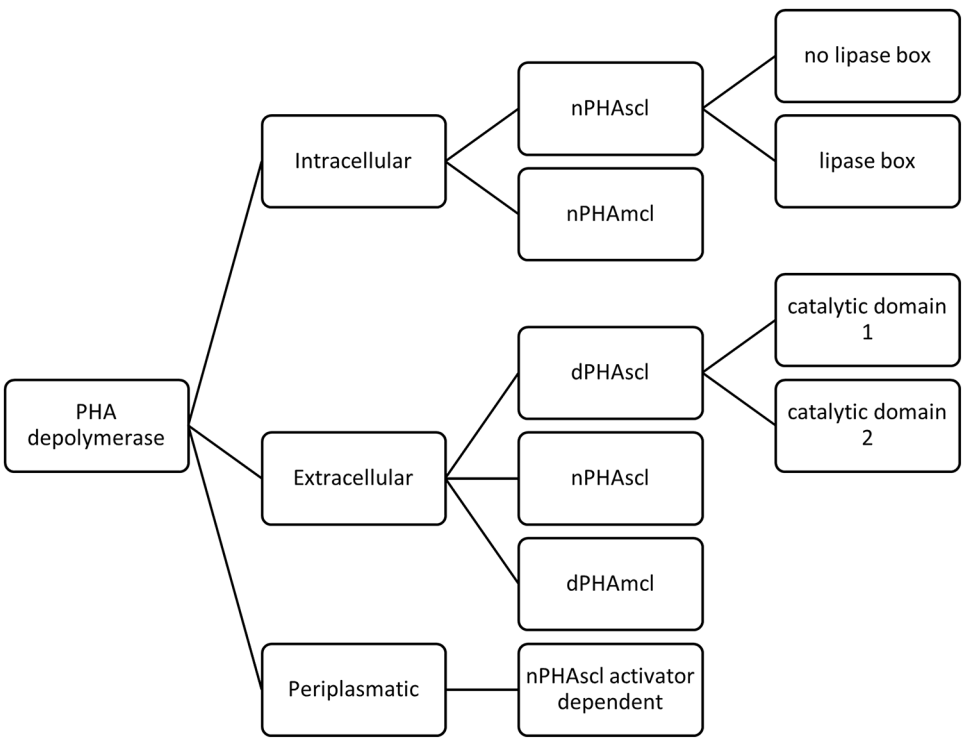


Fig. 6 Classification of PHA depolymerases

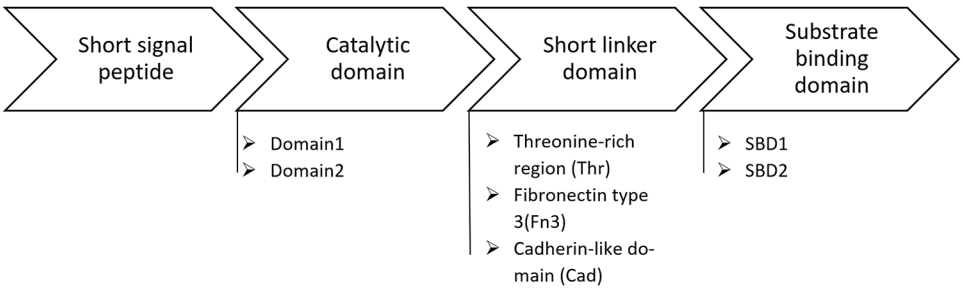


Fig. 7 Regions of a PHA depolymerase and their classifications

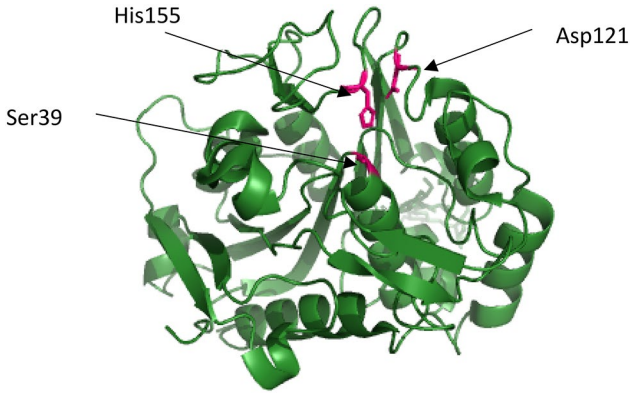


Fig. 8 dPHAscl type II from Penicillium funiculosum. The enzyme has 319 amino acid residues with a molecular mass of 33,569 Da. The enzyme has a α/β hydrolase fold where the catalytic residues are (marked in pink) [172]. Molecule modelled in PyMOL for this work [118]. (**2D80** from Protein Data Bank elucidated using x-ray diffraction)

hydrophobic residues in the enzyme and the methyl groups in the polymer. Check Fig. 10 [179].

Using *Alcaligenes faecalis*, in a study with 5 substrates the SBD was capable of adsorbing on the surface of all films (PHB, PHP, P4HB, P(2HP) and P(6Hx)); However, the enzyme, only hydrolysed 3 films (PHB, PHP and P(4HB) out of the 5, demonstrating that the binding domain works independently of the catalytic domain and that it is less specific [177].

In the other hand, dPHAmcl depolymerases do not contain a substrate binding domain. In these enzymes, the N-terminal region of the catalytic domain is thought to function as substrate binding site [6, 17].

As mentioned before, there are two types of catalytic domains in PHA depolymerase depending on the position of the lipase box and oxyanion hole. Nevertheless, comparisons of substrate specificities between *Alcaligenes faecalis* (domain type 1), *Pseudomonas stutzeri* (type 1),

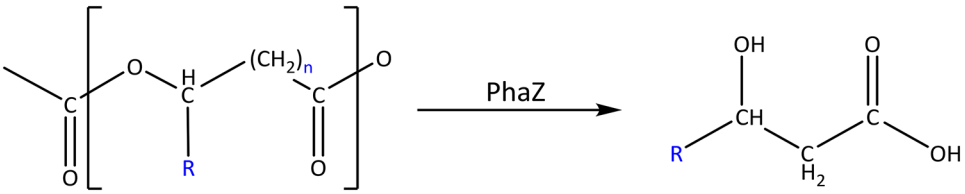


Fig. 9 Polyhydroxyalkanoate polymers are degraded to hydroxyalkanoic acid monomers. Modelled using ChemDraw [62]

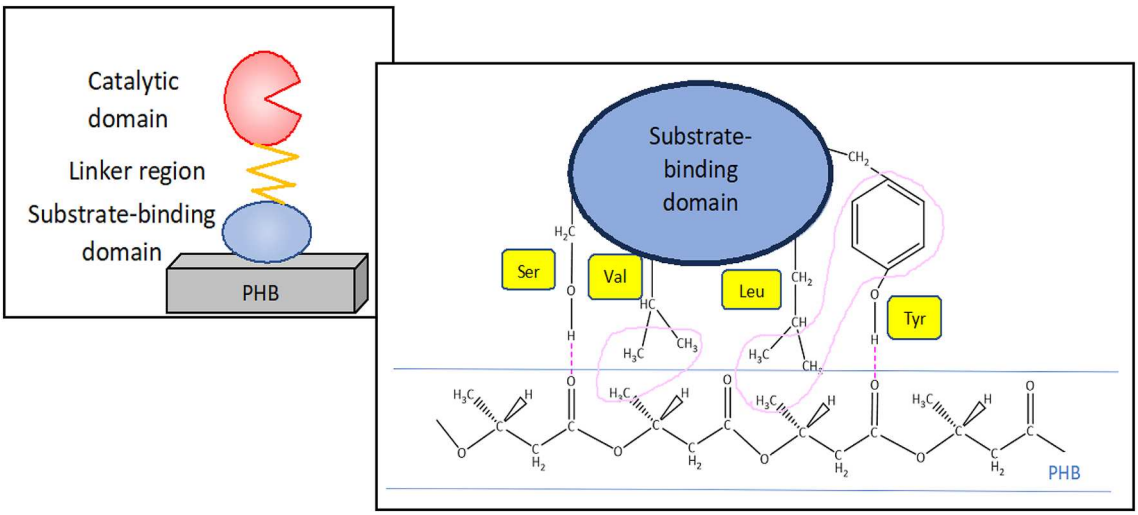


Fig. 10 Schematic model of the interaction of the substrate-binding domain with the PHB (Taken from Hiraishi, 2006 [179]). Dashed bonds are used to represent hydrogen bonds whereas hydrophobic interactions are circled

and *Comamonas acidovorans* (type 2) using 12 different aliphatic polyesters showed that active sites of PHA depolymerases have a similar conformational structure, independently of a difference in the sequence structure of the catalytic domains given that they were able to degrade the same 5 substrates (PHB, PHP, P(4HB), poly(ethylene-succinate) and poly(ethylene-adipate)) and unable to degrade the other seven [180].

The maximum rate of PHA depolymerase catalysed reaction (Vmax) as well as the Km constant for the degradation of PHB at optimal conditions have been measured for several organisms as shown in Table 7. A low Km represents a very big affinity for the substrate.

Protein engineering for catalytic enhancement

Engineering of PHA depolymerases can be achieved using the same techniques described for PHA synthase engineering but a lot less research has been made over PhaZ.

The degradation rate of a piece of PHB is usually on the order of a few months (in anaerobic sewage) or years (in seawater) [185] but genetical engineering can be applied to make the process more efficient, for example, Hiraishi, et al. [186] made an amino acid substitution of the residue Tyr443 for a more highly hydrophobic amino acid (Phe) in PhaZ from *Ralstonia pickettii* which resulted in a higher PHB degradation activity while Tan, et al. [187] performed directed evolution over the catalytic domain through error-prone PCR to achieve a tenfold increase of the PHB depolymerase activity.

Methods to evaluate the enzymatic activity

As mentioned before, one of the most common and simple techniques to identify PHA-producing colonies is staining them with the hydrophobic dye Sudan Black and observing under the microscope. When PHAs consumption conditions are present, colonies expressing intracellular PHA depolymerase will look less stained than before [188], whereas, a simple way to observe extracellular depolymerase activity is using an agar plate assay (halo formation) as indicated by Schirmer, et al. [189]. The diameter of the resulting clear zone semi-quantitatively indicates the activity of the enzyme. The technique can also be used to screen microorganisms that utilize extracellular PHAs [6, 190].

In the other hand, quantitative methods include the measurement of the 3HB released by, either using 3HB dehydrogenase and hydrazine and measuring spectrophotometrically [191, 192] or by directly monitoring absorption at 210 nm of carbonyl groups of 3HB monomer and dimers [193, 194]. Another approach to determine PHA depolymerase activity is to take advantage of the esterase activity of the enzyme and use *para*-nitrophenylalkanoate (pNPA) as substrate. The concentrated p-nitrophenol released by the hydrolysis of pNPB is measured spectrophotometrically at 410 nm [171, 189].

Alternatively, there are weight loss measurements in which PHAs lose weight, over time, as consequence of depolymerization. However, this technique is not practical for rapid routine assays [195, 196].

Finally, non-common methods for recording degradation of polyesters include electron microscopy [197], 1 H-NMR (hydrogen-nuclear magnetic resonance) imaging [198], ^{13}C-NMR (carbon nuclear magnetic resonance) [199] and tracking radiolabelled polymers (this technique can be used to measure both polymerization and depolymerization of PHAs) [200, 201].

Summary of the methods discussed in this section are shown in Table 8.

Lipases capable of degrading PHAs.

It has been proven that several lipases can degrade PHAs [167, 202, 203]. A comparison study of PhaZ from *P. lemoignei* and T1 lipase (isolated from the palm *Geobacillus zalihae*, capable of degrading amorphous P3HB) showed that their active site residues are very well aligned. The enzymes have 21.4% sequence identity and 53.8% sequence similarity only and, yet, their structures are very similar and they both contain the oxyanion pocket and the lipase box pentapeptide (GXSXG) [204], which can explain this phenomenon.

Most bacterial lipases are capable of hydrolyzing polyesters consisting of an ω-hydroxyalkanoic acid such as poly(6-hydroxyhexanoate) or poly(4-hydroxybutyrate), whereas polyesters containing side chains in the polymer backbone such as poly(3-hydroxybutyrate) and other poly(3-hydroxyalkanoates) are not or only somewhat hydrolyzed [167, 203]. Great diversity of commercial lipases going from yeast (*Candida antartica* and *C. rugosa*), bacteria (*Pseudomonas cepacia*, *P. fluorescens*, *Chromobacterium viscosum*), fungus (*Rhizopus arrhizus* and *R. oryzae*), insect (*Myagrus javanicus*), shrub (*Rubus niveus*)

Table 7 PHA depolymerases and their kinetic parameters using PHB as a substrate

Organism	Type	Apparent Km(μg/ml)	Apparent Vmax(μg/min)	
Penicillium expansum	ePHAscl	1.04	4.5	[181]
Thermus thermophilus	edPHAscl-typeI	53	N/A	[171]
Fusarium solani	ePHAscl	100	50	[182]
Penicillium citrinum	ePHAscl	1250	12.5	[183]
Alcaligenes faecalis	edPHAscl-typeI	13.3	N/A	[184]

Table 8 Comparison of common methods to evaluate PhaZ activity

Method	Advantages	Disadvantages	Reference
Sudan Black observation	Easy and quick	Not quantitative result	[188]
Halo formation in plate assay	Easy and quick, for extracellular PhaZ only.	Semi-quantitative result	[6, 189, 190]
Measurement of 3HB release	Quantitative method	No information	[191–194]
Measure esterase activity at 410 nm	Quantitative method	No information	[171, 189]
Measurement of PHAs weight lost	Easy	Not practical for rapid routine assays	[195, 196]

and animals (porcine pancreas) are capable of degrading P(3HB-*co*−4HB) [205–207].

No efforts have been made to enhance the catalytic activity of lipases to degrade PHAs but they have been immobilized on the surface of intracellular polyhydroxybutyrate (PHB) granules by fusion of the lipase gene with the *pha*C operon to produce fatty acid alkyl esters such as biodiesel [208, 209].

Conclusions and future perspectives.

As indicated before in this review, (Fig. 3) the substrate used by PhaC to produce mcl-PHAs is the intermediate of the fatty acid β-oxidation pathway, 3-hydroxyacyl-CoA. Fatty acids of different carbon lengths when fed to the microbial cell are metabolized by this pathway, and each time the cycle is complete the chain loses 2 carbons [210]. As a result, hydroxyacyl-CoA of different chain lengths in the cell can be incorporated to the PHA polymer making the monomer composition very difficult to control.

It has been proven that the deletion of all the copies of certain β-oxidation genes (fadA, fadB and PhaG for example, shown in Fig. 3**)** to interrupt the pathway can lead to the accumulation of predicted homopolymers [211]. In the near future, efforts should be made to synthesize and test homopolymers of different chain lengths and to scale up the process for industrial production. Complementary, the upregulation of the genes fabG, fadE and PhaJ from this same pathway increases the metabolic flux to the production of the PHA substrate 3-hydroxyacyl-CoA thus increasing yield.

Other methods to increase yield and modify PhaC's substrate preference is through protein engineering. In this review we show a list of point mutations that have been proven to change substrate preference and suggest some others that have not been proven yet for future research.

Moreover, in this review we move one step ahead and report the main characteristics, structure, classification and protein engineering methods to modify PhaZ which will be needed in the future to accelerate the degradation of PHA products.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

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