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# Recombinant DNA technology as a tool for improving production of polyhydroxyalkanoates by the natural producers

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## Article info

## Abstract

Polyhydroxyalkanoates (PHAs) are a group of the biodegradable polyesters, and represent an alternative to conventionally used petroleum-based plastics resistant to biodegradation. The production is not cost-competitive compared to conventional plastics, although, there are several bacterial producers capable of PHA accumulating up to 80 % of their cells dry weight using low-cost substrates. PHA production can be improved by transferring specific enzymes or entire metabolic pathways from the most efficient producers to other natural producers. Therefore, the review is focused on genetic modification of bacterial producers, namely the genera *Cupriavidus*, *Pseudomonas*, *Halomonas*, *Aeromonas* and *Bacillus*, for efficient industrial production of PHAs. Recombinant PHA producer can use non-traditional substrates like agro-industrial wastes, namely whey, lignocellulose or glycerol. It is possible to influence the shape and size of the producer's cell by over-expression or knockout of selected genes or to affect its preference for a specific component of a culture medium by modulation of a producer's basal metabolism. The costs of PHA production still be reduced by simplifying the downstream process by enzymatic hydrolysis of selected parts of the cell or blocking the protective mechanisms of the cell against its autolysis caused by the ionic strength of the solution.

## Introduction

Polyhydroxyalkanoates (PHAs) as the microbial storage biopolymers are usually accumulated in response to the absence of a source of macroelements as oxygen, nitrogen, phosphorus, sulfur, iron or magnesium with an excess of carbon. *Cupriavidus*, *Pseudomonas*, *Aeromonas*, *Bacillus*, *Alcaligenes*, *Enterobacter* and certain cyanobacteria and halophilic bacteria produce PHAs under these conditions (Leong *et al.* 2014). Although some producers, namely *Alcaligenes eutrophus, Azotobacter vinelandii* UWD, *Alcaligenes latus* or recombinant *Escherichia coli*, are able to constantly accumulate PHAs without nutrient-limited conditions), but usually accumulate less PHA (Keshavarz and Roy 2010).

The producers usually generate short-chain length (scl) PHAs composed of monomers containing 3 to 5 carbon atoms (*C. necator*, *A. latus*), mainly polyhydroxybutyrate (PHB) or polyhydroxy-valerate (PHV), or medium-chain length (mcl) PHAs composed of monomers consisting of 6 to 14 carbon atoms (*Aeromonas caviae*, *Pseudomonas putida*) such as polyhydroxyhexanoate (PHH), polyhydroxyoctanoate (PHO), polyhydroxy-decanoate (PHD) or polyhydroxydodecanoate (PHDD). PHA producers rarely accumulate long-

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chain length (lcl) PHAs composed of monomers containing more than 15 carbon atoms (Zhang *et al.* 2006; Tan *et al.* 2014b).

The material properties of PHAs are similar to those of conventional synthetic petroleum-based plastics and therefore, represent a suitable alternative made from renewable resources (Tian *et al.* 2005; Berezina and Martelli 2014; Troschl *et al.* 2017). Moreover, these biopolymers are fully biodegradable into carbon dioxide and water by microorganisms from a variety of artificial and natural environment, including wastewater, soil, rivers, lakes, seas, and even hot springs and polar regions (Chen *et al.* 2018; Favaro *et al.* 2019; Tan *et al.* 2020).

Despite the above-mentioned advantages, the cost of PHA production remains high compared to traditional plastics (Koller *et al.* 2013). The aim of the research is therefore to find a fast and cheap way to produce PHAs. Researchers have previously focused on finding a suitable and low-priced substrate for the production of specific PHAs, including the use of by-products derived from a production process as a source of carbon and energy. Current research is concerned with the metabolic improvement of selected species, either in terms of influencing metabolic pathways leading to PHA accumulation, expanding the range of substrates used by the producers or facilitating isolation of PHAs from the cell. In this review, we summarize the latest knowledge about the possibilities of increasing PHA production by genetically modified PHA producers of the genera *Cupriavidus*, *Pseudomonas*, *Halomonas*, *Aeromonas* and *Bacillus*.

## Genetically modified PHA producers

*Genetic modifications for inexpensive carbon sources utilization*

Selection of carbon source is one of the important factors that influence the price of a PHA product. It must be cheap and available in large quantities (Choi and Lee 1997). By-products from various industrial or agricultural productions are strong candidates for this purpose (Koller *et al.* 2005; Solaiman *et al.* 2006). However, a number of PHA-accumulating bacterial producers cannot use them

as a substrate, but this can be changed through genetic engineering.

Whey high in lactose, proteins and lipids as a by-product of milk processing is available in large quantities, but only some strains from the group of PHA producers can utilize it, namely *Hydrogenophaga pseudoflava* DSM1034 (Koller *et al.* 2007), *Methylobacterium* sp. ZP24 (Yellore and Desai 1998) and *Thermus thermophilus* HB8 (Pantazaki *et al.* 2009). Therefore, the induction of the *lacZ* gene coding for *β*-galactosidase was one of the first attempts to expand the range of substrates for PHA production. Povolo *et al.* (2010) disrupted the *phaZ1* (PHA depolymerase) gene of the *C. necator* wild-type strain by the *lacZ* gene replacement with the aim of producing PHAs in a medium with lactose as the sole carbon source. In contrast to the wild-type *C. necator*, the recombinant strain was able to grow and produce PHB in a medium with lactose and whey. PHB accumulation represented 30 % of cell biomass after 48 h in a hydrolyzed whey permeate composed mainly of glucose and galactose, and 22 % of cell biomass after 48 h in a non-hydrolyzed permeate high in lactose. The yield of PHB probably reduced due to non-optimal cultivation conditions. However, due to the low yields of PHAs, research is focused on finding the native PHA-accumulating producers containing lactose-degrading enzyme (Favaro *et al*. 2019).

Molasses is another agricultural by-product of sugarcane or sugar beet processing in the sugar industry. The material contains sucrose usable as the sole carbon source for a PHA producer with the sucrose-degrading enzyme system. If this is not the case, then a gene encoding *β*-fructo-furanosidase, an enzyme that cleaves sucrose to glucose and fructose, can be inserted into the genome of a PHA producer. Park *et al*. (2015) inserted this gene into the *C. necator* wild-type strain to produce high content of PHB (73.2 wt %) from molasses. The conversion of by-products from oil processing has also been achieved with genetically modified strains. An example is the utilization of glycerol as a carbon source for PHA production. Cardozo *et al*. (2019) inserted the *bmgD* gene encoding glycerol-3-phosphate dehydrogenase into the *Bacillus megaterium* PHA producer, and observed

a higher biomass yield and PHA content compared to the wild-type producer.

Lignocellulose is one of the promising widely available and renewable sources of carbon and energy. Hydrolysates of various plants have previously been used as the substrates for PHA production. In hydrolysates, the target substrate for PHA production was glucose as a hydrolytic product released from cellulose. Then, research focused on the utilization of hemicellulose degradation products by incorporating genes required for the consumption of arabinose (Lu *et al.* 2013) or xylose (Liu *et al.* 2014) into *C. necator*. Although these carbohydrates can be used as substrates, lignocellulosic material is generally difficult to use in fermentation processes due to the complicated structure and compounds with a potentially toxic effect on bacterial growth. Phenolic compounds linked to lignocellulose via ester bonds pose a challenge for bioproduct conversion. They are released from lignin as well as hemicelluloses and significantly inhibited the growth of a wide range of microorganisms (Graf and Altenbuchner 2014; Bouarab-Chibane *et al.* 2019). Currently, attempts to assimilate phenols from lignocellulose to valuable products are known. Zhou *et al.* (2020) focused on the use of ferulic acid for PHA production. Although the PHA producer of *P. putida* KT2440 has natural ability to convert ferulic acid to mcl-PHA, the organism's low tolerance to ferulic acid and its toxicity complicate fermentation process. Zhou *et al.* (2020) modified the genome of *P. putida* KT2440 using a CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) genome-editing strategy and via this method, nine genes involved in ferulic acid-to PHA bioconversion were integrated into the *P. putida* genome. The recombinant strain increased the production of PHA to 270 mg.L$^{-1}$ associated with the utilization of 20 mg.L$^{-1}$ ferulic acid from a medium.

*Genetic modifications for production of PHA in co-cultivation system*

Co-cultivation represents an interesting strategy for the simultaneous cultivation of two or more organisms. The metabolic product of the first producer becomes a substrate for another in order to produce the desired metabolite and also utilize a by-product (Goldford *et al.* 2018; Fedeson *et al.* 2020). This concept occurs naturally in the environment, meaning various types of bacteria in communities form a network of interspecies interactions. Therefore, the strategy appears to be a suitable alternative for the utilization of difficult-to-use carbon sources for PHA producers. The co-cultivation system for PHA production usually consists of two bacterial strains (Fedeson *et al.* 2020; Hobmeier *et al.* 2020; Liu *et al.* 2020). Hobmeier *et al.* (2020) tested the possibility of mcl-PHA production in a co-culture of *Synechococcus elongatus* cscB with *P. putida. S. elongatus* fixes CO$_2$ and metabolizes it to sucrose at high salt concentrations and then *P. putida* metabolizes sucrose and accumulates mcl-PHA in the cytoplasm. However, *S. elongatus* requires a nitrate-containing medium for efficient sucrose production and *P. putida* can convert nitrate to ammonium via nitrite that uses as a nitrogen source for its growth. This is a challenge because the PHA production by *P. putida* is more profitable under nitrogen-limited conditions. The recombinant strain of *P. putida* with a deletion of the *nasT* gene lost the ability to convert nitrate as a nitrogen source, but the ability to grow in a medium with ammonium was not affected. The mutant was able to produce up to 8.8-fold higher PHA yield (14.8 %) in the co-culture compared to the wild-type strain.

Co-cultivation system consisting of recombinant bacterial species of *S. elongatus* and *P. putida* was also used in the work of Fedeson *et al.* (2020). The aim was the simultaneous production of mcl-PHAs and the bioremediation of toxic 2,4-dinitrotoluene (2,4-DNT). The recombinant sucrose-uptake *P. putida* (Hobmeier *et al.* 2020) was further modified by insertion of the *dntA* and *dntB* genes from *Burkholderia* sp. R34 allowing the degradation of 2,4-DNT to form non-toxic metabolites of nitrite, pyruvate and propionyl-CoA. This co-cultivation resulted in the degradation of 2,4-DNT associated with the production of mcl-PHA. Although, a lower mcl-PHA yield compared to the results of the other studies (Yang *et al.* 2019; Hobmeier *et al.* 2020) was observed, the advantage is the combination of biopolymer production and bioremediation thus extending the possibility of using co-cultivation strategies.

Liu *et al.* (2020) verified the possibility of co-culture system consisting of recombinant *E. coli* and *P. putida* strains. The production of mcl-PHA was stimulated by the presence of acetate produced by recombinant *E. coli*. Moreover, *E. coli* was modified by knockout of the *ptsG*, *manZ*, *atpFH* and *envR* genes resulting in the preferred carbon source being xylose (not glucose) and *P. putida* had an amplified acetate assimilation pathway by over-expression of acetyl-CoA synthetase and acetate kinase-phosphotransacetylase (Yang *et al.* 2019). The growth of *P. putida* was ensured by glucose as a carbon source and mcl-PHA production by acetate produced by the recombinant *E. coli* strain. The highest mcl-PHA yield was observed by using a 20 g.L$^{-1}$ carbon source (glucose : xylose, 1 : 1; w/w), namely 0.541 g.L$^{-1}$, which is 3.9-fold higher compared to *P. putida* monoculture.

*Morphology engineering for improving PHA production*

Morphology engineering is an interesting approach to increase the efficiency of PHA production without interfering the metabolic pathways of PHA production. This type of engineering affects the morphology of the cell and allows the formation of larger PHA granules, which should also facilitate their isolation. A number of genes are responsible for the shape of the cells, and by modifying them, it is possible to change the shape of the cell from rods to fibers or to form large from small spheres. For example, the *minCD* gene is responsible for cell length, the *ftsZ* gene for the production of cell division protein and the *mreB* gene for cytoskeleton protein production (Jiang and Chen 2016; Elhadi *et al.* 2016; Zhao *et al.* 2019).

Over-expression of the *mreB* gene led to the formation of larger cells compared to the control (Wu *et al.* 2016), but over-expression of this gene associated with knockout of the *minCD* gene increased mcl-PHA production in *P. mendocina* NKU (Zhao *et al.* 2019). However, this strategy was more successful in the process of producing PHB by the recombinant *E. coli* strain (Elhadi *et al.* 2016). Tan *et al.* (2014a) observed that the inducible over-expression of the *minCD* gene during the stationary growth phase of *Halomonas* TD08 elongated cell shape, leading to 1.2-fold higher accumulation of PHB in the cells. Another advantage of modifying the cell shape was the formation of intertwined fiber network during the production of PHB. This network allowed the self-flocculation and precipitation of cells, simplifying the subsequent downstream processing. Jiang *et al.* (2017) developed a temperature-sensitive plasmid expression system containing the *mreB* or *ftsZ* genes and introduced this construct into *H. campaniensis* cells. Recombinants are able to grow at 30 °C and their growth is comparable to the wild-type strain. Morphological changes such as cell size expansion or cell shape elongation did not appear until the temperature was raised to 37 °C and the temperature-sensitive plasmid was no longer replicated in the recombinants. This led to an increase in PHB yield, which reached up to 80 % cell dry weight. The results of the above-mentioned research show a new interesting strategy for the industrial PHA production by combining the expression and/or knockout of various metabolic and morphological genes.

It is possible to influence not only the cell morphology but also the morphology of the PHA granules themselves. The *phaP1* gene is responsible for regulating the size and number of PHA granules. Knockout of this gene resulted in the production of PHA granules up to 10 μm accumulated in the intracellular space of recombinant *Halomonas bluephagenesis* TD01 cells. The resulting size was limited only by the size of the cell itself (Shen *et al.* 2019).

*Genetic modifications for improving PHA biosynthesis*

PHB synthesis is one of the most studied metabolic pathways of PHAs. It consists of three basic enzymatic steps including enzymes such as PhaA (*β*-ketothiolase), PhaB (NADPH-dependent acetoacetyl-CoA reductase) and PhaC (PHA synthase) (Fig. 1). PhaA catalyzes the condensation of two acetyl-CoA molecules to acetoacetyl-CoA that is converted to (*R*)-3-HB-CoA ((*R*)-3-hydroxybutyryl-CoA) by PhaB. In the final step, PhaC polymerizes (*R*)-3-HB-CoA into PHB. PhaC belongs to the key enzymes for PHA biosynthesis and catalyzes the polymerization of hydroxyacyl-

CoA thioesters. The other PHA biosynthetic pathways differ mainly in the enzymes forming various hydroxyacyl-CoA thioesters for final polymerization by PhaC.

*β*-oxidation of fatty acids is an alternative PHA biosynthetic pathway for the formation of hydroxy-acyl thioesters (Fig. 1). The products of this pathway are mainly mcl-PHAs due to the length of formed precursors. The key enzymes are FadE (acyl-CoA dehydrogenase) and PhaJ (enoyl-CoA hydratase). FadE catalyzes the reaction of acyl-CoA to enoyl-CoA and PhaJ uses enoyl-CoA as a substrate. Some PHAs can be synthesized from (*R*)-3-hydroxyacyl-CoA obtained from 3-ketoacyl-CoA using FabG (3-ketoacyl-CoA reductase) (Philip *et al.* 2007).

The third PHA biosynthetic pathway involves two important enzymes, namely PhaG (3-hydroxyacyl-ACP-CoA transferase) and FabD (malonyl-ACP transacylase) (Fig. 1). The substrates are converted to (*R*)-3-hydroxyacyl-ACP and then to (*R*)-3-hydroxyacyl-CoA by PhaG and thus to PHA by PhaC (Philip *et al.* 2007; Laycock *et al.* 2013). The involvement of above-mentioned enzymes in PHA biosynthesis associated with important metabolic pathways such as Calvin, ribulose monophosphate (RuMP) or Krebs cycle, glycolysis as well as pathways for biosynthesis and degradation of amino acids and fatty acids (Lu *et al.* 2009).

Bacteria can accumulate PHAs composed of different monomers depending on the carbon source used (Noda *et al.* 2005), with PhaC playing a key role in this process. The PhaC enzymes with different specificity can use variable precursors for PHA synthesis. PhaC can be classified into one of four classes (I – IV) based on primary structure, substrate specificity, and subunit composition (Rehm 2003; Pötter and Steinbüchel 2005). Recently, Tan *et al.* (2020) discovered a new PhaC isolated from the Antarctic *Janthinobacterium* isolate, which could be included in the new fifth class.

Over-expression of the *phaC* gene generally leads to higher yields of PHAs. Akdoğan and Çelik (2020) over-expressed the native *phaC* gene in *B. megaterium*, resulting in increased biomass yield and PHA content compared to the wild-type strain in both batch and fed-batch cultivation processes. The use of the *phaC* gene isolated from other PHA producers may lead to the incorporation of the new hydroxyacyl-CoA thioester monomers to the growing PHA chain, and moreover, knockout of the original *phaC* gene and incorporation of the new *phaC* gene can cause the formation of a completely new PHA. After insertion of the *phaC* gene from *Pseudomonas* sp. 61-3 into *C. necator*, the mutant was able to accumulate copolymer P(3HB-*co*-3HA) with a relatively high 3HB content (Matsusaki *et al.* 2000a; Matsusaki *et al.* 2000b; Matsumoto *et al.* 2001). Cha *et al.* (2020) tested the effect of four different *phaC* genes from *Thiocapsa pfennigii*, *C. necator* H16, *Chromobacterium violaceum*, and *Paracoccus denitrificans* on PHA production by *P. putida* EM42. Knockout of the *phaC1* and *phaC2* native genes in all four mutants resulted in the production of P(3HV-*co*-4HV) copolymer instead of the naturally produced mcl-PHA consisting of 3HD and 3HO. The most common ratio of the accumulated scl-PHA was 60 : 40 (w/w) for 3HV and 4HV, respectively and 3HB content was only about 1 %. The mutant with the *PhaC* gene from *P. denitrificans* produced a copolymer with a ratio of 3HV : 4HV 96 : 4 (w/w). This synthase showed lower substrate specificity for 4HV compared to other synthases.

Insertion of the *phaC* gene from another producer may be accompanied by a negative effect on the type of produced PHA. The *Aeromonas hydrophila* mutant with the *phaC* gene from *Pseudomonas stutzeri* 1317 accumulated PHA consisting of C4-C12 monomers with a dominant amount of 3HHx instead of P(3HV-*co*-4HH) (Liu *et al.* 2011). Tan *et al.* (2020) used the *C. necator* mutant with the *phaC* genes from Antarctic *Janthinobacterium* isolates. No accumulation of PHA was observed in the first case and in the second case, the mutant accumulated only 18.7 wt % of P(3HB). The higher PHB content was determined in biomass growing at 20 °C compared to 30 °C. The key factor influencing the choice of synthase source is both the specificity of the enzyme and the differences of cultivation conditions between the PHA producer and the *phaC* source.

The other important enzymes for PHB biosynthesis are PhaA and PhaB responsible for the preparation of precursors for the synthesis of PHB chain (Philip *et al.* 2007). If PhaA and PhaB expression is too

**Fig. 1.** Metabolic pathways of PHA production (modified from Choi *et al.* 2020). Legend: 3HA-CoA – 3-hydroxyacyl-CoA; 3HB – 3-hydroxybutyrate; 3HP – 3-hydroxypropionate; 3HV – 3-hydroxyvalerate; 4HbD – 4-hydroxybutyrate dehydrogenase; AldD – aldehyde dehydratase; BktB – *β*-ketothiolase; Cat2 – 4-hydroxybutyryl-CoA transferase;  CoA – coenzyme A; DhaB – glycerol dehydratase; DhaT – 1,3-propanediol dehydrogenase; FabA – 3-hydroxyl-ACP dehydrase; FabB – *β*-ketoacyl-ACP synthase I; FabD – malonyl-CoA:ACP transacylase; FabF – *β*-ketoacyl-ACP synthase II; FabG – 3-ketoacyl-ACP reductase; FabH – *β*-ketoacyl-ACP synthase III; FabI – enoyl-ACP reductase; FadA – 3-ketoacyl-CoA thiolase; FadB – 3-hydroxyacyl-CoA dehydrogenase; FadD – acetyl-CoA synthetase; FadE – acyl-CoA dehydrogenase; HadA – isocaprenoyl-CoA:2HIC CoA-transferase; Pcs' – propionyl-CoA synthetase; Pct – propionyl-CoA transferase; PduP – propionaldehyde dehydrogenase; PhaA – *β*-ketothiolase; PhaB – acetoacetylCoA reductase; PhaC – polyhydroxyalkanoate synthase; PhaD – PHA-polyhydroxyalkanoate depolymerase; PhaG – 3-hydroxyacyl-ACP-CoA transferase; PhaJ – enoyl-CoA hydratase; PrpE – propionyl-Coa synthetase; Sbm – methylmalonyl-CoA mutase; SucD – succinate semialdehyde dehydrogenase; YgfG – methylmalonyl-CoA decarboxylase.

low and there are no other sources of metabolic precursors for PHA synthesis, over-expression of the *phaC* gene does not lead to an increase in biopolymer accumulation (Liu *et al.* 2011). The activity of PhaA and PhaB should be checked before modifying the producer's *phaC* gene. Over-

expression of the *phaA* and *phaB* genes in the *A. hydrophila* mutant caused 85 % increase in copolymer accumulation compared to the wild-type strain. Knockout of the acetic acid pathway was also beneficial for this strain resulting in further 47 % higher accumulation of PHA copolymer due to the prevention of acetic acid formation (Liu *et al*. 2011).

Unlike Liu *et al*. (2011), Shi *et al*. (2020) did not reduce acetic acid production by deleting certain genes in the acetic acid pathway, but used this acid as a carbon source to produce HBT in the *A. hydrophila* mutant with over-expression of the *phaA* and *phaB* genes. Moreover, over-expression of enzymes involved in the acetic acid pathway caused further 6-fold higher PHB content compared to that produced by control strain without this modification. The high activity of PhaA and PhaB caused the accumulation of a sufficient intracellular concentration of 3-hydroxybutyryl-CoA, a key intermediate for PHB synthesis by PhaC. Subsequent over-expression of *phaC* led to further increase in PHA accumulation to 19.82 % of cell biomass. This is the first study to report the production of PHB by engineered *A. hydrophila* in the acetate-propionate medium.

PhaB uses NADH or NADPH to catalyze its reaction. The most of PHB-accumulating bacteria use NADPH dependent PhaB (Ling *et al*. 2018). NADPH is a typical cofactor for the redox reaction of anabolism. Selected species of bacteria such as *Allochromatium vinosum*, *Azotobacter beijierinckii* and *H. bluephagenesis* have NADH dependent PhaB. NADH plays a key role in respiration or anaerobic fermentation processes at high or low oxygen concentrations, respectively (Sauer *et al*. 2004). NADPH-dependent PhaBs are dependent on the availability of a reduced form of NADPH, which is consumed in any anabolic process in the cell and therefore, a significant accumulation of PHA occurs under essential element-limited conditions (N, P, S, Fe or Mg) with an excess of carbon (Steinbüchel 1991). At the same time, NADH is used exclusively within respiration or fermentation. Ling *et al*. (2018) demonstrated that *H. bluephagenesis* produced higher concentrations of NADH under oxygen-limited conditions. Blocking the *etf* operon limited electron transfer from NADH, a lack of oxygen increased

the $NADH/NAD^+$ ratio, resulting in an increase PHB content to 90 % of dry weight. PHB production under oxygen-limiting conditions represents a significant reduction in energy consumption due to the possibility of carrying out fermentation with a lower agitation rate and less aeration (Ling *et al*. 2018). The problem of NADH excess inhibiting pyruvate metabolism and the tricarboxylic acid (TCA) cycle was solved by adding acetic acid to the culture medium. NADH-dependent PhaB consumes NADH during the reduction of acetic acid to 3HB-CoA. Moreover, the addition of the acetic acid increased both the biomass yield and the PHB content.

Production of new copolymers can be achieved by replacing the *phaB* gene with genes involved in other metabolic pathways of PHA production. Jung *et al*. (2019) prepared the recombinant *C. necator* strain by deletion of the *phaB1*, *phaB2* and *phaB3* genes and over-expressing a synthetic operon consisting of *phaC2* from *Rhodococcus aetherivorans*, *phaA* and *phaJ* from *Pseudomonas aeruginosa*. The recombinant strain produced the terpolymer P(3HB-co-3HV-co-3HHx) (42 % of dry weight) in a medium containing volatile fatty acids such as propionate and butyrate instead of P(3HB). Yu *et al*. (2020) deleted the native *phaC* gene in *H. bluephagenesis* and expressed the *phaC* and *phaJ* genes from *A. hydrophila* and endogenous *fadD* (acyl-CoA synthetase). The mutant produced copolymer P(3HB-co-3HHx) with the high content of 3HH (35 %).

FadE and PhaJ play a key role in *β*-oxidation of fatty acids. Although FadE represents a rate-limiting step in *β*-oxidation of fatty acids, it has received little attention (Eggers and Steinbüchel 2013; Magdouli *et al*. 2015). PhaJ is one of the more studied enzymes because its insertion or modification makes it possible to obtain scl-mcl copolymers using a recombinant *C. necator* strain with more advantageous physical properties, such as flexibility and elasticity, compared to commonly produced PHB (Noda *et al*. 2005). Flores-Sánchez *et al*. (2020) constructed a recombinant *C. necator* with an inserted *phaJ1* gene from *P. putida* KT2440 and a *fadE* gene from *E. coli* K12. Activation of the *fadE* gene caused the higher biomass yield and PHA accumulation than the original strain. The *phaJ1* gene from *P. putida* was selected because of its high affinity for 3HHx

and 3HO substrates. Its activation led to the implementation of 3HO and 3HD into the formed scl-mcl copolymer. This result supports the hypothesis that PhaC from *C. necator* can incorporate also mcl monomers into the PHA chain if suitable metabolic precursors are available.

Deletion of *phaG* and *phaZ* genes involved in *β*-oxidation of fatty acids causes the production of mcl-PHA with an increased content of 3HD or 3HDD monomers. Zhao *et al.* (2020) prepared the *P. mendocina* NK-01 mutant with deleted *phaG* and *phaZ* genes. Deletion increased the amount of 3HD (94.7 mol %) and 3HDD (68.7 mol %) in a medium containing sodium decanoate and sodium dodecanoate.

FadD allows efficient conversion of lipids to PHA through fatty acid metabolism (Fig. 1). Liu *et al.* (2011) observed that co-expression of the *vgb* (*Vitreoscilla* hemoglobin gene) and *fadD* (*Escherichia coli*) genes in *A. hydrophila* 4AK4 led to the production of homopolyester P(3HV) (47.74 %).

The *Vitreoscilla* hemoglobin (VHb) is an important oxygen-binding protein. VHb is induced at low concentration of oxygen in wild-type producers, effectively binding the oxygen and supplies it to the terminal respiratory oxidases, leading to respiration under oxygen-deficient conditions (Webster 1988). This mechanism affects the production of PHA by bacteria such as *E. coli*, *A. hydrophila* and *P. putida* (Liu *et al.* 2011; Duarte de Oliveira *et al.* 2020). Tang *et al.* (2020) expressed the *vgb* gene in *C. necator* together with the knockout of the *ldh* gene (*L*-lactate dehydrogenase). The mutant showed higher productivity and PHB content and lower production of fermentation by-products compared to the wild-type strain in low oxygen culture medium. Ouyang *et al.* (2018) expressed low-oxygen inducible VHb in PHA producers of *H. bluephagenesis* TD01 and *H. campaniensis* LS21. Both mutants achieved more intensive biomass growth and higher PHB production. However, Duarte de Oliveira *et al.* (2020) observed that the production of VHb did not mitigate the negative effect of oxygen limitation. The efficiency of VHb engineering must be assessed for each producer. There is a possible explanation for these different results. While Duarte de Oliveira *et al.* (2020) focused on the producers of mcl-PHA derived from precursors

of *β*-oxidation of fatty acids, Tang *et al.* (2020) worked with the producers of scl-PHA derived from precursors of glycolysis. Duarte de Oliveira *et al.* (2020) also observed the lower biomass yield and PHA content compared to the wild-type strain. These results suggest that an incorrectly selected metabolic modification may cause an increase in the metabolic load of the cell, resulting in a decrease in PHA production.

3-hydroxyacyls are commonly produced, but it is also possible to enrich the biopolymer with 4-hydroxyacyls by inserting the *orfZ* gene (4HB-transferase) responsible for the conversion of 4HB to 4HB-CoA. The *H. bluephagenesis* mutant with the *orfZ* gene from *Clostridium kluyveri* with the T7-like expression system (MmP1) produced copolymer P(3HB-*co*-4HB) and the total content of the copolymer was 63 % of dry weight (Chen *et al.* 2017). Shen *et al.* (2018) were also able to increase the accumulation rate of this copolymer up to 80 % by using a more suitable promoter.

*Genetic modifications to avoid PHA degradation*

The intracellular activity of PHA depolymerase represents another challenge in PHA production. The enzyme is activated in the absence of a carbon source and degrades the accumulated PHA granules in the cell, resulting in the release of carbon and energy that the producer can use for its growth (Steinbüchel and Hein 2001). These enzymes are present on the surface of PHA granules (Rehm 2003) and, therefore the inactivation of intracellular PHA depolymerase by genetic engineering appears to be necessary to increase PHA recovery.

Povolo *et al.* (2015) deleted the *phaZ1* gene encoding PHA depolymerase in the *C. necator* wild-type strain and their results suggest an increase in PHB accumulation during bacterial growth. However, knockout of the *phaZ3* gene had no statistically significant effect on PHB accumulation, suggesting that the *phaZ3* gene is unlikely to play a role in PHB degradation (Povolo *et al.* 2010). Kobayashi and Kondo (2019) also observed 2.9-fold higher PHB production by *Rhodobacter sphaeroides* HJ mutant with knockout of *phaZ*. Moreover, knockout of this gene associated with over-expression of PHB biosynthesis genes (*phaA3*, *phaB2* and *phaC1*) caused 3.9-fold higher PHB production compared

to the wild-type strain. Similar results have been reported by other authors focusing on the inactivation of mcl-PHA depolymerases (de Eugenio *et al.* 2007; Cai *et al.* 2008).

It should be noted that knockout of PHA depolymerase is not always associated with increased PHA recovery. Zhou *et al.* (2020) observed that the incorporation of the *phaC1* gene in *P. putida* KTc9n13 associated with disruption of the *phaZ* gene did not cause a significant increase in PHB accumulation. Also, Vo *et al.* (2015) did not observe a significant effect on increasing PHA production by damaging the *phaZ* gene in *P. putida* KT2440. Deletion of this gene caused 1.2-fold lower mcl-PHA production than wild-type strain. Salvachúa *et al.* (2020) did not observe a significant effect on PHA production by disruption of the *phaZ* gene in *P. putida* KT2440. Different results of these studies could be due to various cultivation conditions and genetic variability (difference of genotypes, deletion of the selected *phaZ* gene) (Zhou *et al.* 2020).

*Genome reduction for improving PHA production*

Genome reduction is a process in which appropriate deletion of selected genome fragments can increase producer growth, broaden the substrate specificity, improve energy metabolism, and increase the product yield and the genome stability (Fehér *et al.* 2007; Chi *et al.* 2019). The process eliminates a large proportion of the genome which is not important for the growth and production of PHA but consumes a lot of energy and carbon (Wang *et al.* 2018).

The *P. mendocina* NK-01 mutant with 14 large fragments removed and 7.7 % genome reduction accumulated 11-fold higher mcl-PHA content than the wild-type strain (Fan *et al.* 2020). Deletion of 6 genes involved in the β-oxidation of fatty acids of *P. medocina* caused an increase of the dominant 3HD monomer to 90.01 mol % and total mcl-PHA content to 58.6 mol %. Moreover, this modification increased the cell shape due to the higher accumulation of PHA granules in the intracellular space (Zhao *et al.* 2020). *P. putida* KT2440 with 4.12 % reduction of the total genome size increased the amount of mcl-PHA up to 1.5-fold compared to the *P. putida* wild-type. Knockout of *gcd* gene

(glucose dehydrogenase gene) in the *P. putida* mutant caused a further 22.17 % increase in PHA content (Liang *et al.* 2020). Wang *et al.* (2018) deleted genes involved in the production of flagella and pili in the *P. putida* KT2442 genome. The genome reduction by 1.2 % resulted in accelerated mutant growth, no flagella production, reduced biofilm production and 45.6 % higher PHA production than the original strain.

*Genetic modifications for simplify PHA isolation*

Down-stream processes affect the total cost of PHA production. The choice of isolation and purification steps is also related to the application of the biopolymer, because some steps may alter PHA properties or add undesirable biomass-derived compounds such as lipoproteins or toxins to the final product (Koller *et al.* 2013). Modification of the producer's genome by molecular biology tools may facilitate the process of isolating PHA granules from the cell (Borrero-de Acuña *et al.* 2017; Poblete-Castro *et al.* 2020) or reduce the number of isolation steps (Boynton *et al.* 1999; Rodríguez Gamero *et al.* 2018).

In recent years, the goal is to develop a programmable cell lysis system that allows the isolation of intracellular metabolites by autolysis of the production organism (Hajnal *et al.* 2016; Tamekou Lacmata *et al.* 2017; Borrero-de Acuña *et al.* 2017). Hori *et al.* (2002) used the phage holin-endolysin system in *B. megaterium* to release PHA from cells. Ultimately, approximately two-thirds of the accumulated PHA was released from the cells after induction of lysis by xylose. Cell lysis lasted approximately 30 h. Martinez *et al.* (2011) used a similar system inducible by 3-methyl benzoate in *P. putida* KT2440. It allowed the disruption of approximately 86 % of the cells within 15 h and facilitated the subsequent extraction of PHA. Borrero-de Acuña *et al.* (2017) used toluic acid as the inductor in the phage holin-endolysin system. Although the use of the toluic acid inducer in *P. putida* KT2440 reduced the biomass by 25 % during cultivation, mcl-PHA content was maintained at 30 %. After lysis, 95 % of the bacterial cells were disrupted and approximately 75 % of the total biopolymer was recovered during 16 h. Hajnal *et al.* (2016) tested the implementation of the Lambda

lysis system works in *H. campaniensis*. Spontaneous autolysis was observed after centrifugation of the cells and prolonged passive standing. The use of the lytic system has some disadvantages such as relatively long lysis time (15 – 30 h) and low amount of polymer released (75 - 86 %) (Hori *et al.* 2002; Martinez *et al.* 2011; Borrero-de Acuña *et al.* 2017).

Another possibility is to modify the mechanisms responsible for maintaining cell wall integrity. OprF (porin F) is a protein responsible for the permeability and integrity of the cell wall (Chevalier *et al.* 2017), OprE (porin E) increases the diffusion of small ions (Jaouen *et al*. 2006), MscL (mechanosensitive channels of large conductance) acts as a safety valve opening pores in sudden hypo-osmotic shock, what is the last protection of the cell against their lysis due to osmotic shock (Colombo *et al.* 2003). Poblete-Castro *et al.* (2020) modified this system in *P. putida* by over-expressing the OprF and OprE, and deleting MscL. Due to hypertonic and hypotonic treatment, 95 % of the cells were lysed within 3 h, with 94 % mcl-PHA yield.

High-pressure homogenization (HPH) is one of the most widely used methods for cell lysis and is also one of the environmentally acceptable methods. However, its use releases large amounts of DNA, which increases the viscosity of the solution and complicates subsequent purification procedures (Ling *et al.* 1998; Tamer *et al.* 1998; Van Wegen *et al.* 1998). The decrease of viscosity is usually achieved by expensive processes, such as heat treatment or the addition of hypochlorite or commercially available nucleases (Koller *et al.* 2013). However, it is possible to insert the nuclease gene into a natural producer of PHA to reduce the viscosity and the cost of the downstream process. This method was also used by Boynton *et al.* (1999), which inserted the *nuc* gene encoding a nuclease from *Staphylococcus aureus* into natural PHA producers such as *C. necator* and *P. putida.* No negative effect on PHA growth and production was observed for both PHA producers. It was recorded the lower viscosity of the solution compared to wild-type strains treated with a commercial nuclease. The highest nuclease activity was observed for *P. putida*. Rodríguez Gamero *et al.* (2018) inserted the *nuc* gene into two native PHA producers, *C. necator* DSM 545 and *Delftia acidovorans* DSM 39. The gene was expressed in both producers, and the inserted gene did not affect the amount of produced biomass. In both cases, a higher amount of PHB was reported. The nuclease activity was higher in the *C. necator* mutant; the recombinant was used in the next experiments. The lower viscosity of the solution after HPH was observed compared to the original strain.

## Conclusion

Genetic engineering of native PHA producers appears to be a suitable way to reduce the cost of PHA production. Molecular biology techniques can be used to broaden the range of substrates suitable for the production of PHAs, such as whey, molasses or crude glycerol. Morphology engineering as a new strategy for the construction of efficient microbial cell factories makes it possible to obtain larger and easier isolatable PHA granules from modified cells. Over-expression of selected genes involved in the PHA synthesis or knockout of the genes for specific metabolic pathways are associated with several advantages, such as increased PHA production, expansion of the range of produced biopolymers with different material properties adaptable to the intended purpose. Genetic modification also provides various ways to increase the number of monomers for PHA synthesis. The co-cultivation of two or more recombinant bacterial species appears to be promising due to cost reduction as well as the utilization of by-products. The overall resulting cost of PHAs can be reduced by a suitable isolation method through the implementation of selected self-destruction mechanisms based on the lytic system of bacteriophages or by porins. A key aspect of PHA production is use of by-products that presents an alternative to products primarily intended for human consumption. Production of PHAs can be introduced as a part of future biorefinery concept.

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# Conflict of Interest

The authors declare that there is no conflict of interest.

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