Biotechnology Advances 42 (2020) 107582

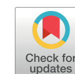
Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Current trends in the production of biodegradable bioplastics: The case of polyhydroxyalkanoates



João Medeiros Garcia Alcântara[a], Francesco Distante[b], Giuseppe Storti[b], Davide Moscatelli[a], Massimo Morbidelli[a], Mattia Sponchioni[a,*]

[a] Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, via Mancinelli 7, 20131 Milano, Italy
[b] Institute for Chemical and Bioengineering, Department of Chemistry and Applied Biosciences, ETH Zurich, Vladimir-Prelog-Weg 1, 8093 Zurich, Switzerland

ARTICLE INFO

Keywords:
Polyhydroxyalkanoates
Bioplastics
Microbiological
Fermentation
Enzymatic
Chemical
Ring-opening polymerization
Production
Biodegradable

ABSTRACT

The global pollution caused by plastics and microplastics is stimulating intense research towards more environmentally friendly materials, preserving the remarkable application characteristics of the currently available polymers. Among these, polyhydroxyalkanoates (PHAs) have been hailed as the solution to replace conventional, oil-based plastics. Given their biodegradable nature and mechanical properties, their use can be envisioned in a wide range of applications reducing the environmental footprint. Several types of processes have been proposed for their production, which can be grouped in three main classes: (i) microbiological, (ii) enzymatic and (iii) chemical processes. Given the significant amount of literature available on this topic, this review aims to critically analyse what has been proposed so far in each of these classes, with specific reference to their potential to provide bioplastics that can actually replace the currently available materials. A comparison is made, based on the following aspects: achievable molecular structures (such as molecular weight and composition distributions), raw-material and production costs and availability of large-scale production technologies. Finally, some considerations and ideas on what should be further investigated and implemented to realize the economically sustainable production of PHA are brought forward.

1. Introduction

Amidst concerns derived from the overuse of fossil-based plastics, there is their resistance to degradation, which leads to large accumulations of waste materials in the environment. This has stimulated scientific and financial efforts on finding green alternatives to the traditional plastics. Among these, bio-based polymers have the potential to substitute traditional polymers, ensuring similar properties, while being biodegradable and thus reducing the environmental footprint related to anthropogenic activities (Raza et al., 2018).

A class of biopolymers that is currently attracting much attention, due to the wide range of mechanical and thermal properties it can access, is the one of polyhydroxyalkanoates (PHAs). PHAs are linear polyesters, whose general structure is schematized in Fig. 1D. The possibility of tuning their properties arises from the side chain *R* and the length of the alkyl chain *n*, which are characteristic of the monomer used for the synthesis, besides their chain length, x.

PHAs are naturally occurring polymers, mainly produced by bacteria for carbon and energy storage (Możejko-Ciesielska and Kiewisz, 2016). Specifically, more than 150 monomeric building blocks have been identified as having potential as substrates for PHA synthases and hence for polymerization. However, most of these have only been used in laboratory settings and solely a handful have been adopted by industry. Interestingly, most building blocks are chiral and since PHA synthases are stereospecific only *R*-configured PHAs are found in nature (Koller et al., 2013b).

Each HA monomer has a side chain (R), often a saturated alkyl group. However, this chain can also be constituted of unsaturated alkyl, branched alkyl and substituted alkyl groups (Tan et al., 2017). PHAs are classified according to the number of carbons in this side chain. Less than 5 carbons correspond to short-chain length PHAs (scl-PHAs), between 5 and 14 to medium-chain length (mcl-PHAs) and with more than 14 carbons, to long-chain length PHAs (lcl-PHAs). However, lcl-PHAs are not very common in nature (Raza et al., 2018).

In general, PHAs are biodegradable, biocompatible, non-toxic, insoluble in water and soluble in chloroform and other chlorinated solvents. Their glass transition temperature varies from −50 to 4 °C and the melting temperature from 40 to 180 °C, according to their chemical composition and chain length. However, depending on the type of PHA, composition and molecular weight, these properties vary greatly, even

* Corresponding author.
E-mail address: mattia.sponchioni@polimi.it (M. Sponchioni).

https://doi.org/10.1016/j.biotechadv.2020.107582
Received 16 April 2020; Received in revised form 8 June 2020; Accepted 18 June 2020
Available online 02 July 2020
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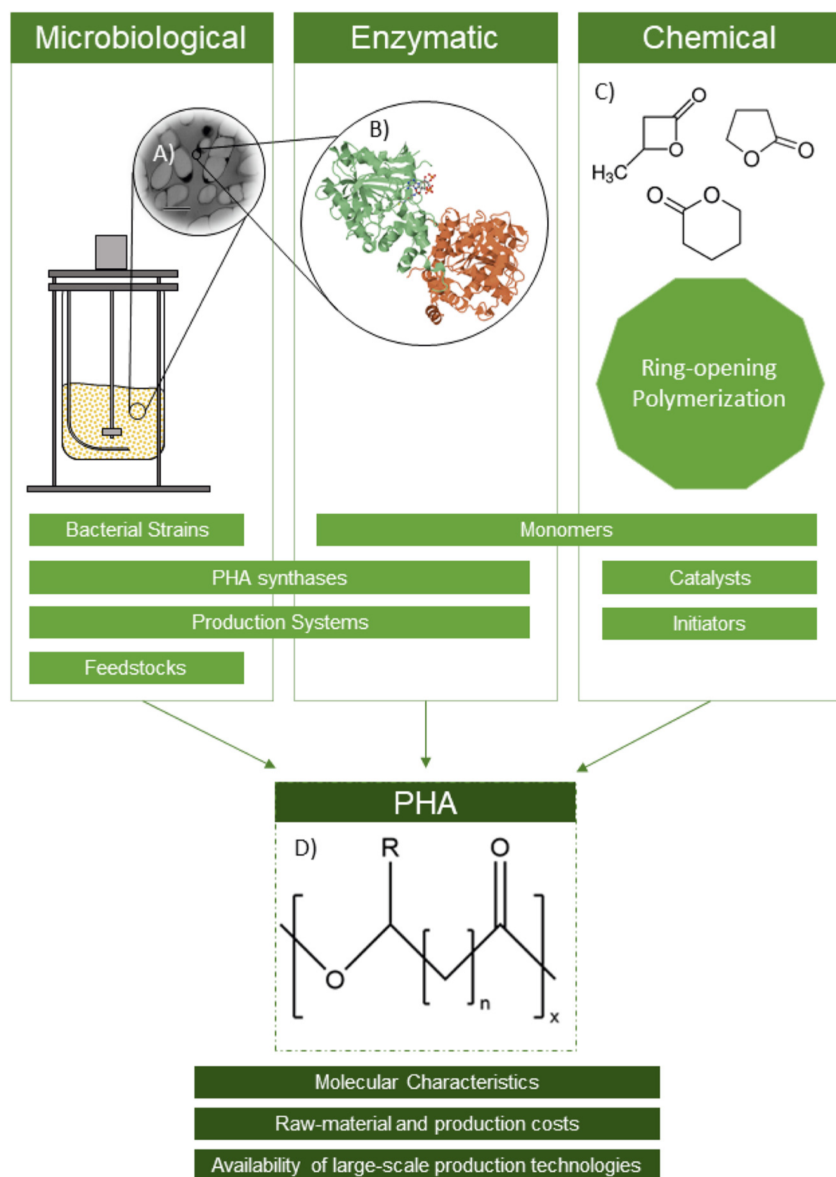

Fig. 1. Main processes for the production of PHA: microbiological, enzymatic and chemical. Light green boxes indicate the factors which most affect each of the classes of processes. Dark green boxes indicate the three main topics for the critical comparison present in this work. A) Transmission electron micrograph of bacterial cells accumulating very large quantities of PHA as inclusion granules (white bodies inside the cellular membrane). Bar represents 0.5 µm. Reproduced with permission from Sudesh et al., 2000 (Sudesh et al., 2000). B) Crystal structure of the PHA synthase (PhaC) from Chromobacterium sp. USM2 bound to Coenzyme A. Image of 6K3C (rcsb.org) (Chek et al., 2020) ("Jmol: an open-source Java viewer for chemical structures in 3D, 2020.") C) Examples of lactones which can be polymerized into PHAs by ring-opening polymerization (ROP) (from left to right: β-butyrolactone, γ-butyrolactone, δ-valerolactone). D) General chemical structure of PHAs: R represents the side chain, n the length of the alkyl chain and x the length of the polymeric chain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

affecting the degradation kinetics of the material (Raza et al., 2018). Therefore, the modulation of the chemical microstructure of these polymers can be viewed as a powerful tool for the tuning of their mechanical and thermal properties, thus accessing materials suitable to a wide variety of applications, possibly replacing conventional plastics.

These polymers were observed for the first time in 1888. However, at that time, their composition and biological role could not be properly defined. As early as 1926, poly-3-hydroxybutyric acid (P(3HB)) was obtained from *Bacillus megaterium* by a French scientist, even if its function as carbon and energy storage was established only in 1959. One year later, the commercialization of PHA started (Możejko-Ciesielska and Kiewisz, 2016). Typically, commercial PHAs have been produced through a microbiological route (Tan et al., 2017). However, both chemical and enzymatic processes have been widely investigated as a strategy to improve the control of the physicochemical properties of the product (Tang and Chen, 2018; Thomson et al., 2009).

Due to the increasing interest towards this new class of bioplastics and the growing literature on the different strategies for its production, the present work aims at reviewing the recent advances in the production processes of PHAs. The three main routes, namely microbiological, enzymatic and chemical, schematically illustrated in Fig. 1, are considered and their main features, potential scalability and current hindrances towards commercialization are discussed. These production routes are then compared side-by-side in terms of

i) molecular characteristics, such as the molecular weight (MW) and composition distribution of the products
ii) raw-materials and production costs
iii) availability of industrial technologies for large-scale production

These considerations will then highlight the issues that need to be addressed to facilitate the affirmation of PHAs in the plastic market.

2. The microbiological route

The production of PHAs is intrinsically connected to natural strategies for survival deployed under environmental stress by bacteria, which form inclusion bodies as carbon and energy storage (Możejko-Ciesielska and Kiewisz, 2016). The insoluble granules can take up 90% of the dry weight of the cell mass (see Fig. 1A). This strategy is widely employed by gram-positive and gram-negative bacteria and, also, in archaea. Representative examples include *Cupriavidus necator* (previously known as *Ralstonia eutropha*) and *Pseudomonas sp.* (Sudesh et al., 2000). Besides the storage of energy, this process allows for increased

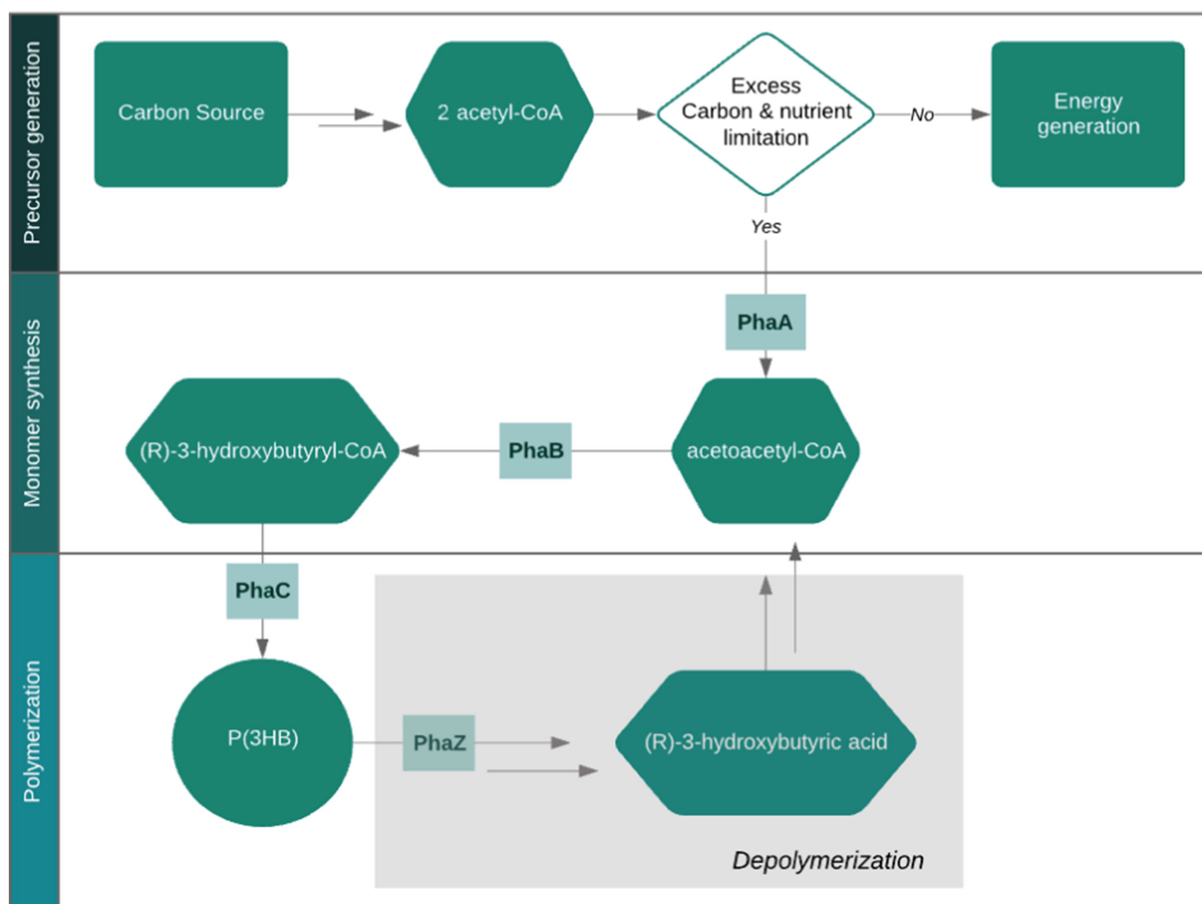

Fig. 2. Simplified metabolic pathway for the production of P(3HB). PhaA: β-ketothiolase. PhaB: NADPH-dependent acetoacetyl-CoA reductase, PhaC: PHA synthase, PhaZ: PHA depolymerase.

stress tolerance to transient environmental conditions, such as heat, osmotic shock and ultraviolet radiation (Tan et al., 2014). Although this strategy is naturally occurring in a wide variety of microorganisms, alike many other bioproducts, it is also possible and advantageous to genetically alter non-PHA producers (e.g. *Escherichia coli*) to obtain very efficient PHA producers, as discussed in details later in this work (Możejko-Ciesielska and Kiewisz, 2016).

2.1. Metabolic pathways

The carbon source from which the PHA is produced can be catabolized through a number of metabolic pathways (Meng et al., 2014). As a representative example of the PHA class, this section will deal with the specific case of P(3HB), which is currently the most synthetized PHA worldwide (Vandi et al., 2018). For this biopolymer, the final steps are invariably driven by three different enzymes: PhaA, PhaB and PhaC (see Fig. 2). In this case, the carbon source is transformed into acetyl-coenzymeA (CoA), 2 molecules of which are converted into acetoacetyl-CoA by a thiolase (β-ketothiolase, PhaA). The precursor for this type of PHA, (*R*)-3-Hydroxybutyryl-CoA is then formed by the NADPH-dependent acetoacetyl-CoA reductase (PhaB), leading to the polymerization of P(3HB) by the PHA synthase (PhaC) (Sudesh et al., 2000).

This type of metabolite production is deeply linked to the central metabolic pathways of the bacteria, including, but not limited to: glycolysis, Krebs cycle, β-oxidation, de novo fatty acid synthesis, amino acid catabolism, Calvin cycle and serine pathway. For example, Acetyl-CoA is an intermediate which is shared by these pathways and the production of PHA. In fact, in most native PHA producers, the formation of PHA only occurs in certain environmental conditions due to the metabolic flux of acetyl-CoA. When there is a surplus of carbon and there is no other growth-related limitation, the synthesis of considerable amounts of CoA from the Krebs cycle blocks PHA synthesis by inhibiting PhaA. Acetyl-CoA is in fact assimilated into the Krebs cycle for energy production and cell growth. However, if the carbon surplus conditions are maintained and there is a limitation of an essential nutrient, the CoA concentration becomes non-inhibitory, leading to the supply of acetyl-CoA to the PHA biosynthetic pathways (Tan et al., 2014). Based on this metabolic causality network, these conditions are mimicked in bioreactors in order to produce PHA. Following the normal procedure for producing secondary metabolites (i.e. metabolites not associated with the cellular growth stage), most PHA producing processes consist of two steps. The first one is the growth stage, which aims to increase the cell concentration inside the reactor, where there is no nutrient limitation. This is followed by the production stage, where a limitation is introduced (e.g. lack of nitrogen source), in order to direct the metabolic pathways to the accumulation of PHA and to stop growth and cell multiplication processes (Koller et al., 2018).

It should be pointed out that, after PHA accumulation, if these limitations are removed, the PHA can be depolymerised and the normal metabolism of the bacteria for energy production and growth is re-established. Under this change in conditions, a PHA depolymerase (PhaZ) breaks down the PHA into easily assimilated energy compounds (Sudesh et al., 2000).

There is a wide variety of possible substrates and ensuing metabolic pathways for the production of PHAs, which cannot be discussed here in detail. For this, the interested reader is directed to the comprehensive review by Steinbüchel and Lütke-Eversloh (Steinbüchel and Lütke-Eversloh, 2003).

2.2. PHA synthases

PHA synthases catalyse the polymerization of CoA thioesters of HA into PHA polymers, releasing the CoA moiety (Zou et al., 2017). In general, this enzyme is denominated PhaC, since it is encoded by the gene *phaC*. This gene is normally clustered with other genes that are necessary for the production of PHAs, namely *phaA* and *phaB*, which encode the β-ketothiolase and the NADPH-dependent acetoacetyl-CoA reductase, respectively (Zou et al., 2017).

Generally, PHA synthases can be divided into 4 classes (Rehm, 2003; Tsuge, 2016). Class I and class II include enzymes constituted by only one type of subunit (PhaC), with molecular masses between 61 and 73 kDa. Both classes utilize specific substrates. Class I synthases, present e.g. in *C. necator*, preferentially use CoA thioesters of several (*R*)-3-hydroxy fatty acids with 3 to 5 carbon atoms. On the other hand, class II synthases prefer CoA thioesters of various (*R*)-3-hydroxy fatty acids with 6 to 14 carbon atoms (Rehm, 2003).

Both the remaining classes are constituted by two distinct types of subunits, one of which they have in common: the PhaC subunit. This has an amino acid sequence with a similarity of 21–28% to class I and II synthases and molecular mass of about 40 kDa. The other subunit is designated PhaE (approximately 40 kDa) and PhaR (approximately 20 kDa), for class III and IV, respectively. These synthases have a substrate specificity towards CoA thioesters of several (*R*)-3-hydroxy fatty acids with 3 to 5 carbon atoms (Rehm, 2003).

As expected, based on the exhibited substrate specificity, synthases from classes I, III and IV produce scl-PHAs, while enzymes from class II produce mcl-PHAs.

Representative species are as follows: *Cupravidius necator* (previously *Ralstonia eutropha*), *P. aeruginosa, Allochromatium vinosum* and *Bacillus megaterium*, for class I, II, III and IV, respectively (Tsuge, 2016). The nucleotide sequences of at least 59 PHA synthases have been obtained. As such, some of these do not conform to the aforementioned classes. Examples include the PHA synthases obtained from the following microorganisms: *Thiocapsa pfennigii, Aeromonas punctate* and some *Pseudomonas sp.*(Rehm, 2003). Interestingly, a PHA synthase from an extremely halophilic arcaebacterium was identified and characterized, possibly constituting a new class of synthases. This enzyme had its maximum activity at 40 °C, and was stable up to 60 °C, where it decreased by only about 10% (Hezayen et al., 2002; Rehm, 2003).

With regards to the molecular mass, the PHAs produced by class I enzymes exhibit a higher molecular mass than those obtained by class II, normally, between 500 kDa to several millions and from 50 kDa to 500 kDa, respectively. Class III and IV synthases produce PHAs with intermediate molecular masses (Rehm, 2003).

2.3. Catalytic mechanism

The model that the current literature suggests for the polymerization of PHAs is based on the PHA synthase-catalysed polymerization reaction that includes 3 steps: initiation, propagation and termination (Tsuge, 2016). Fig. 3 describes these steps from a chemical standpoint using a class I synthase, as an example. This type of synthase has only one type of subunit, PhaC, which after translation, exists as water-soluble single forms. In the initiation step, two PhaC subunits are dimerized, thus forming the active enzyme. There are two proposed mechanisms for this process, involving two thiol groups (Stubbe and Tian, 2003; Tsuge, 2016). The difference lies in the origin of these groups: in one of the models the thiols are provided by the cysteine residues from each of the PhaC subunits in the dimer. In the other one, one thiol is provided by a cysteine residue and the other by the thioester bond present in the monomer.

The exact catalytic mechanism for this step has been researched for more than 30 years (Sagong et al., 2018), however no definitive answer has been found to date. Nonetheless, significant insight has been uncovered in the last years with the determination of the crystal structure of PhaC from *Chromobacterium sp.* USM2 (Chek et al., 2017) and *Cupriavidus necator*, the latter by two distinct research groups (Kim et al., 2017; Wittenborn et al., 2016). Specifically, they found that the distance between the catalytic cysteine residues present in each of the subunits of PhaC was too large to allow for the ensuing chemical reactions that should occur in the first model. Hence, they favour a catalytic mechanism with only one catalytic site. In spite of this, no agreement has been found on the exact enzymatic mechanism that occurs at the single catalytic site.

Two opposing models have been proposed. On the one hand, Kim et al. postulated a single active site non-processive ping-pong mechanism (also called by other authors, as a processive mechanism with a single tunnel) (Kim et al., 2017), in which the ingress to and egress from the active site is done via the same tunnel. On the other hand, in the processive mechanism (or processive mechanism with in-out tunnels) (Chek et al., 2017; Teh et al., 2018; Wittenborn et al., 2016) a through passage exists and there is the covalent bonding of the polymeric chain to the cysteine residue present in the active site. A comprehensive overview of these putative mechanisms can be found in the work by Sagong et al. (Sagong et al., 2018).

Very recently, Chek et al. have brought forward the crystal structure of the catalytic domain of PhaC of *Chromobacterium sp.* bound to CoA (Chek et al., 2020). This is significant since it allows further understanding into the dynamics of the dimerization process. Explicitly, it was found that PhaC undergoes a conformational change when bound to CoA, forming an asymmetric dimer with a closed and an open conformation for each of the subunits. In addition, this research group postulates that this conformation eases both the entrance of substrates to the active site and the formation of an egress tunnel for the product, adding evidence to support the processive mechanism with in-out tunnels. However, it should be pointed out that this PhaC-CoA crystal structure has still not been determined for *C. necator*, which should provide further insight into the discussed enzymatic mechanisms.

In any case, the polymerization process will continue with the propagation phase, in which, the polymeric chain grows by the addition of monomers. Finally, chain termination occurs when the PHA synthase reaches the limit of its catalytic activity and becomes inactive (Tsuge, 2016). With this regard, Tian et al. (Tian et al., 2005) claim that the synthase can "sense" the size of the outgoing chain and catalyse the movement of the polymeric chain to another amino acid, while leaving a primed monomer in its place, ready to restart the polymerization of a new chain. In alternative, a chain transfer (CT) reaction can also occur. In this case, the polymer chain is transferred to a CT agent, which covalently binds to the carboxy terminal of the polymer chain. Possible CT agents include water, 3HB and other hydroxy compounds (Tsuge, 2016).

It should be noted that the polymerization carried out by the PHA synthase can be regarded as a living polymerization. Su and co-workers (Su et al., 2000) investigated this system and found evidence that the molecular weight of the final biopolymer is closely related to the monomer to enzyme ratio. In particular, when reaching total conversion, the number average molar mass was uniquely defined by this ratio. In addition, by running two of such reactions, one after the other, they obtained a di-block copolymer - also a characteristic of living polymerizations. This is in spite of the MW distribution of the produced polymers being somewhat broad. This is indeed unusual for living polymerizations, which are often characterized by polydispersity smaller than about 1.5. However, such broadening can be explained by the fact that in these systems the rate of initiation is considerably slower than that of propagation.

2.4. Microbiological strains

The selection of the microbiological strain is one of the points of prime importance for the successful production of the desired PHA. This choice directly impacts the type of substrate used, as well as the

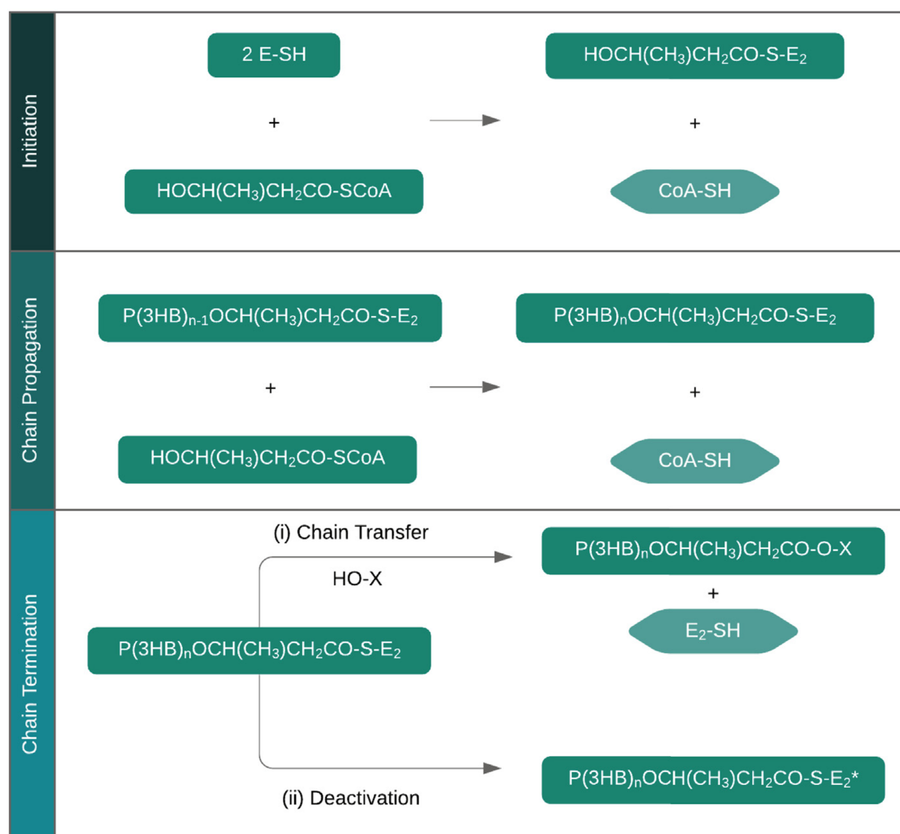

Fig. 3. Model proposed by Tsuge (Tsuge, 2016) for the enzymatic polymerization of P(3HB). CoA-SH – free coenzyme A, E-SH – PHA synthase subunit, E_2-SH – dimerized PHA synthase (active), E_2-SH – dimerized PHA synthase (inactive), HO-X – chain transfer agent, 3HB – 3-hydroxybutyryl monomer.

molecular weight and molecular structure of the final polymer, in addition to other physico-chemical properties (Wang et al., 2014). In addition, it can also affect the process for isolating and purifying the produced PHA due to structural differences of the corresponding cells (Koller et al., 2013a).

The ability to produce PHA is a somewhat diffused attribute in microorganisms, having been identified in more than 70 bacterial and archaeal genera. In particular, scl-PHAs are produced by a wide range of bacteria, e.g. *Cupriavidus necator*, while mcl-PHAs are mainly produced by *Pseudomonas sp.* (Możejko-Ciesielska and Kiewisz, 2016). As mentioned earlier, the variety of PHA-producing strains also entails the myriad of carbon catabolic pathways and PHA anabolic pathways, generating a huge diversity of substrates from which PHA production can occur (Tan et al., 2014).

In general, for biotechnology purposes, an industrial production strain should possess the following characteristics: not be a pathogen and not produce toxins, have a clear genomic background, be of easy genetical manipulation, have a fast growth and a wide substrate utilization. If possessing such characteristics, most likely the production strain can be manipulated to increase the levels of PHA accumulation and substrate conversion and even the cell size. All of this enables the augmentation of the amount of polymer that each cell can accumulate and facilitates the rupture of the cellular membrane and hence the removal of the PHA granules (Tan et al., 2017; Wang et al., 2014).

Native PHA-producing bacteria can be genetically engineered to increase their polymer production. Species like *Cupriavidus necator* can accumulate PHAs in satisfactory amounts, and was, in fact, one of the first ones used at an industrial level (Możejko-Ciesielska and Kiewisz, 2016). Specifically, accumulations have been reported until 89% of cellular dry weight (CDW) (Tan et al., 2014) and productivities of at least 3.1 g/(L h) (Koller et al., 2018). For a comprehensive comparison of the accumulation and productivities of the different microorganisms producing PHA the reader is directed to the review by Tan et al. (Tan et al., 2014).

As mentioned above, non-native bacteria can be modified to produce PHA. Here, *Escherichia coli*, the “work-horse” of biotechnology, is indeed one of the most used. This organism possesses several advantages: the possibility of using a variety of cheap carbon sources, no intracellular PHA depolymerase and a very well-known genetic background. These characteristics establish this species as one of the most promising organisms for the large-scale production of PHA. Specifically, the production of PHA in *E. coli* entails the transfer of a PHA synthase structural gene from a native PHA producer, the assurance of the expression of the active form of the enzyme and the engineering of pathways to provide sufficient concentration of the precursors (Li et al., 2007). This species has in fact been genetically engineered to produce PHAs up to 90% of the CDW (Lee and Chang, 1995). Chen and Jiang have published a detailed review on the genetical manipulation of bacteria to increase PHA production (Chen and Jiang, 2017). In addition to the increased PHA accumulation, non-native PHA producers as *E. coli* have the potential to narrow the molecular weight distribution of the produced polymer. Polydispersity indexes as low as 1.4 have been achieved, very similar to the values obtained with an in vitro PHA polymerization (Leong et al., 2014; Tsuge, 2016).

In general, several factors related to the chosen strain can impact the characteristics of the produced PHA, and in particular the following ones impact directly the molecular weight (Rehm, 2003; Tsuge, 2016):

i) The level of expression of active PHA synthase protein in the cells. The higher the concentration, the lower the molecular mass.
ii) The degradation of PHA during biosynthesis, such as the availability of enzymes that hydrolyse PHAs, including PHA depolymerases, but also of other unspecific esterases and lipases. If these enzymes are not present, polymers with higher molecular masses

can be produced.

iii) The catalytic activity of the active PHA synthase.

iv) Occurrence of chain transfer reactions (see section 3.2).

Interestingly, new bacterial strains have been discovered which produce PHAs extracellularly. These include *Pseudomonas Corrugata* and *Pseudomonas Mediterranea* (Licciardello et al., 2019). While, currently, the yields are still low, this could be an interesting option, since it would remove the extraction stage from the PHA production process, with significant cost reductions.

2.5. Feedstocks

The choice of the substrate is of paramount importance for at least two reasons. Firstly, it can represent up to 50% of the overall production cost (Raza et al., 2018). Secondly, it is probably the most important factor determining the polymer/*co*-polymer composition. Since different carbon sources are metabolised through distinct metabolic pathways, they produce different PHA monomers (Leong et al., 2014). In addition, there is also an ethical consideration: the selected feedstock should not compete with food and feed related sources. This means that the use of the so-called 2nd-generation feedstocks is strongly encouraged, to prevent the rise of prices of the global food supply (Jiang et al., 2016; Koller and Braunegg, 2018). Also, for this reason, research is focused on reducing the cost of production by using various waste streams as carbon source. These include: whey, starch, spent coffee grounds, wastewaters, wheat and rice straw (Raza et al., 2018), glycerol (Koller and Marsalek, 2015; Phithakrotchanakoon et al., 2015), and lignin (Kumar et al., 2019), among others. These research efforts also are fully in line with the concept of a biorefinery, where the PHA production is integrated into the manufacture of other products and bioplastics are produced to valorise waste streams (Kumar et al., 2017). However, one major concern in using these strategies for PHA production is the necessity to assure constant characteristics of the polymerization feedstocks. This appears, in fact, to be a prerequisite to prevent producing biopolymers with non-reproducible characteristics, such as changing molecular mass and copolymer composition (Raza et al., 2018). Also, this may impact some end applications of the PHA. For example, in the case of medical applications, the PHA produced from waste materials may contain impurities (Raza et al., 2018) that could harm the biocompatibility of the produced plastics (Koller et al., 2013a).

2.6. Process-related factors

Several factors related to the production process itself also affect the quality and the chemical composition of the polymer. For example, Penloglou et al. (Penloglou et al., 2012) investigated the effect of key operating variables, such as nutritional and aeration conditions, on the molecular weight of the resulting polymer, through the development of a metabolic model of the microorganism coupled with a kinetic model of the polymerization steps. They found that even the feeding policy has the potential to modify this polymer characteristic.

Temperature is another process variable that influences the molecular weight of the produced PHA. Cultures of *E. coli* JM109 ($phaRC_{BSP}$) operated at 37 °C and 25 °C showed that the MW of the PHA decreases with the culture time at the higher but not at the lower temperature. This behaviour was explained by the occurrence of significant random scission of the polymer chains at 37 °C. However, this conclusion was not confirmed when synthases from other sources were heterologously expressed in *E. coli*. The authors claimed that this behaviour was then unique to this synthase extracted from *Bacillus* sp. INT005 (Agus et al., 2010; Leong et al., 2014). Actually, other related studies found that for the production of ultra-high MW PHA, higher temperatures, combined with a slightly acidic medium, contribute to the polymer chain elongation (Leong et al., 2014). This qualifies as evidence that more than any other factor, it is the genetic background that affects the most the characteristics of the produced polymer.

In the above mentioned review of Leong et al. (Leong et al., 2014), the production of ultra-high MW PHA using different *E. coli* strains was compared. They found large differences among the different strains and that even the way the plasmid was constructed and its bacterial origin affected the MW and polydispersity of the polymer, as well as its composition in the case of copolymers.

Currently, all the industrial PHA producers either adopt batch or fed-batch bioreactors. However, lately there has been some interest in the utilization of continuous processes (Koller et al., 2018). This is driven by the potential of such processes to increase productivities and reduce the plant footprint for the same PHA production. Stand-alone chemostats have been considered to produce PHA (Zinn et al., 2003). However, since PHA is a secondary metabolite, that is its formation does not occur concomitantly to cell growth, it is not possible to have in the same reactor optimal conditions for cell growth and limiting conditions for stimulating PHA production. For this, the use of cascades of continuous stirred tank reactors (CSTRs) appears better suited. For example, Atlić et al. have realized a 5-stage CSTR cascade, where the first one is used for cell growth, while the others operate under nitrogen limitation, so as to induce PHA accumulation. Using this process a productivity of 1.85 g/(L h) and a PHA accumulation of 77% of CDW (Atlić et al., 2011) were achieved. This aspect is well discussed in the comprehensive review by Koller (Koller et al., 2018).

Concluding, albeit centred around genetic makeup of the selected PHA producer, various factors can influence the productivity and the characteristics of biosynthetic PHA, including molecular weight and composition distributions. Although the amount of data is still limited, it is clear that these factors strongly affect the thermo-mechanical properties and workability of the resulting polymer.

3. The enzymatic route

In vitro PHA production has been hailed as having the potential to increase control over the polymer characteristics, such as molecular weight and *co*-polymer composition, compared to the biological route, while reducing costs by separating the PHA synthesis from bacterial growth (Thomson et al., 2009). However, this process closely depends on the supply of high-quality synthase, monomers and other adjuvants for enzymatic reactions, which can have elevated costs (Rehm, 2010). In particular, the production of substantial amounts of synthase has been the main obstacle to the large-scale application of this process. Specifically, extraction from the native host is hampered due to aggregation and current production involves over-expression and purification in heterologous recombinant organisms, such as *E. coli* (Thomson et al., 2009).

Also, monomer production can be highly laborious due to the costs and complexity of producing an enantiomerically pure compound and in its active form, meaning that CoA needs to be chemically linked to the produced monomer. Although PHA polymerase is absolutely enantio-selective, the supply of only the *R* enantiomer to the enzyme is critical. Tajima and co-workers (Tajima et al., 2009) found, in fact, that the molecular weight of the polymer was reduced when both enantiomers were in solution. It was postulated that the *S* monomer, although not reactive, binds to the catalytic active site of the synthase, which then cannot further activate the addition of *R* monomers. There are several chemical routes for the production of the monomer. However, they all lack the ability of producing only the *R* enantiomer. Noyori and co-workers employed an asymmetric hydrogenation of keto esters, producing the monomer with an enantiomeric excess of 99.4% (Noyori et al., 1987). Huang and co-workers (Huang and Hollingsworth, 1998) also developed a method, starting from a lactone, for the production of 3-hydroxy acids with similar enantiomeric excess, which could be applied to the production of the 3HB monomer, however it is rather complex. There have also been reports of a synthetic

route to produce enantiomerically pure 3HB-CoA, however this involves 9 steps and exhibits overall yields below 35% (Jia et al., 2016).

Processes suitable for commercial applications should start from widely available and less expensive substrate and perform an entirely enzymatic process. For example, Tokiwa et al. reported the conversion of acetoacetate into (*R*)-3-HB by the (*R*)-3-HB dehydrogenase with the addition of NADH and H^+ (Tokiwa and Ugwu, 2007). Nevertheless, the adjuvants still need to be furnished, possibly including, besides CoA, ATP and NADPH (Thomson et al., 2009). Another downside is created by the concomitant release of CoA as the polymerization progresses. As it was mentioned above, CoA can, in fact, have an inhibitory effect on PhA and PhaC (Zou et al., 2017). Several processes have been developed to counteract these disadvantages. One option is to reduce the CoA consumption, by recycling it into the system several times. One example of this involves the use of an acyl-CoA synthase and has the advantage of allowing the strict control of monomer composition in the polymerization of PHA *co*-polymers, through the ratio of monomers in solution. However, the disadvantage of this system is that free CoA remains in solution, inhibiting the activity of the PhaA (Satoh et al., 2005). With a view to solve this problem, a two-phase reaction system was developed which also included a mechanism for CoA recycling (Han et al., 2009; Tajima et al., 2004). For this mechanism, the system relies on an ester exchange reaction between CoA in the water phase and acetyl thioester of ethyl thioglycolate (AcETG), leading to the formation of AcCoA in the water phase. Through the use of a propionyl CoA transferase, the AcCoA reacts with the chemically synthesised monomer (3HB), leading to the formation of the activated monomer, 3HB-CoA. This is, in turn, polymerized by a PHA synthase into P(3HB) (Han et al., 2009).

Another interesting possibility would be to immobilize the enzyme enabling its reuse. Several established industrial processes make use of immobilized catalysts, such as the production of high fructose corn syrup (glucose isomerase) and the transesterification of food oils (lipase), among others (DiCosimo et al., 2013). However, despite several authors arguing that it should be possible to immobilize the PHA synthase, to our knowledge this result has not yet been achieved (Thomson et al., 2009).

4. The chemical route

The most used strategy to chemically synthetize PHA is the ring-opening polymerization (ROP). This is a living polymerization employing a metal catalyst and an initiator (usually an alcohol) for the polymerization of cyclic ester monomers, or lactones, into linear polyesters. Given the living nature, the average chain length can be modulated through the ratio between the monomer and the initiator. In addition, the molecular weight distribution is usually narrow (polydispersity < 1.5), provided that transesterification reactions are avoided during the process. This strategy enables, through the proper choice of the monomer, to synthesize a wide variety of homo and *co*-polymers (Hori et al., 1999, 1995; Tang and Chen, 2018). Table 1 shows examples of various lactones that can be used to produce different kinds of PHA homopolymers.

The ROP processes described in the literature make use of different catalysts, such as β-diiminate zinc alkoxide complexes (Rieth et al., 2002), lanthanide or yttrium complexes (Hong and Chen, 2016a; Tang and Chen, 2018), magnesium dibutyl catalysts (Hong and Chen, 2016a), organic catalysts (Hong and Chen, 2016a; Lohmeijer et al., 2006; Makiguchi et al., 2011), tert-Bu-P_4 base (Hong and Chen, 2016b), distannoexane catalysts (Hori et al., 1999, 1995) and magnesium and zinc complexes (Liu et al., 2010). In addition to homopolymers, due to its the living nature, ROP is very convenient to produce also block copolymers. With respect to the reaction mechanisms, we believe that all of the processes referred to in Table 1 occur through a coordination-insertion mechanism (Hong and Chen, 2016a; Tang and Chen, 2018). The interested reader can refer to the review by Lecomte and Jérôme (Lecomte and Jérôme, 2012) to further deepen the chemistry of these systems.

It should be noted that all the ROP processes mentioned so far either make use of enantiomerically pure lactones or produce the atactic polymer. Since the challenge is to reproduce the naturally occurring polymer, while using a synthetic process with better economics, the racemic mixture of the lactone should be used, whilst guaranteeing the production of (*R*)-PHA. Following this concept, Tang & Chen (Tang and Chen, 2018) proposed a different process, starting from the eight-membered cyclic diolide, which can be obtained from bio-sourced dimethyl succinate, and using an yttrium racemic salen complexes-based stereoselective catalyst. The high molecular weight isotactic polymer described in the last row of Table 1 was obtained.

Actually, the ROP of lactones can also be catalysed enzymatically. Gorke et al. (Gorke et al., 2007) used the lipase B enzyme from *Candida Antarctica* and produced different PHAs with molecular weights up to 8.1 kg/mol and yields in the order of 85%. However, this process suffers from all the drawbacks related to the cost and availability of the enzyme discussed in the previous section about the enzymatic route.

5. Critical comparison of the different production processes and future trends

In order to properly compare the three different routes proposed so far for the production of PHA, the following factors should be taken into account: (i) molecular characteristics of the polymer, (ii) raw-material and production costs and (iii) availability of large scale production technologies.

When considering the quality of PHAs, the most important parameters are the molecular weight distribution and the tacticity, since these determine the final mechanical properties of the biopolymer.

In terms of MW, the microbiological route has set the gold standard, at least with respect to the order of magnitude of the maximum average value of the MW, which is in the range of 10^4 to 10^5 g/mol for native bacteria (Tan et al., 2014). Although this is already high with respect to many applications, even higher values have been obtained through genetic engineering modifications, such as rearranging of metabolic pathways or eliminating the presence of PHA depolymerases. Currently it is possible to achieve MW as high as 10^7 g/mol (Sudesh et al., 2000). Similar values have been achieved also through enzymatic reactions. This is not surprising since these reactions are more easily controlled and exhibit an intrinsically living character, as discussed above. However, this does come at a cost: complex processes and expensive raw materials. While bacteria produce PHA with similar characteristics in a very efficient manner, without requiring elaborate upstream strategies and using cheap carbon sources, enzymes need expensive raw materials such as the monomer and CoA, in addition to other chemicals needed to realize the chosen production system. It has been estimated that the production cost of PHB through enzymatic reactions is in the order of 286,000 USD per gram, against the 0.0025 USD per gram requested by the microbiological route (Rehm, 2010). Such a huge difference, although it may be somehow overestimated, makes the enzymatic route of interest only for purely scientific investigations. With this respect, the chemical production route is similar to the enzymatic process. On the one hand, the raw materials are cheaper than the enzymatic ones, since only lactones are used. On the other hand, due to the occurrence of transesterification reactions, the polydispersity is often greater than 2 (see Table 1). Since the chemical route tries to mimic the PHA produced through fermentation methods (which is invariably (*R*) configured), tacticity also comes into the play as an additional issue. The limited availability and the high cost of enantiomerically pure lactones, which are necessary to grant isotacticity, add on to the production costs.

About the polydispersity, *Đ*, the situation is quite different. The chemical and enzymatic processes almost always produce very narrow molecular weight distributions, due to the fact that side reactions are almost completely absent and the process is highly controlled. This is

Table 1
Production of several types of polyhydroxyalkanoates via ROP of diverse lactones. M_n: number average MW, Đ: polydispersity. AA^{Pip}: (E)-2,6-Diisopropyl-N-(2-((2-(Piperidin-1-yl)Ethylimino)Methyl)Phenyl)Aniline, n.d.: non determined.

Raw material *R* or *S*		M_n (kg/mol)	*Đ*	Conv. (%)	Catalyst	Initiator	Prod. Poly.		Ref.
Type	Stereochemistry						Type	Tact.	
β-butyrolactone	*racemic*	64.3	1.06	90	Complex (AA^{Pip})ZnEt	9-AnOH	P(3HB)	(*S*/*R*)	(Liu et al., 2010)
		27.5	1.12	99					
		311	2.27		1-ethoxy-3-chlorotetrabutyldistannoxane	–			(Hori et al., 1993)
	(*R*)	178	2.38					(*R*)	
γ-butyrolactone	*racemic*	30.2	2.40	29	$La[N(SiMe_3)_2]_3$	$C_6H_4(CH_2OH)_2$	P(4HB)	n.d.	(Hong and Chen, 2016a)
		11.5	1.8	90					
		36.8	1.82	13.9	KH	–			(Hong and Chen, 2016b)
		25.0	2.94	90	tert-Bu-P_4	BnOH			
δ-valerolactone		27.5	1.08	90.7	Diphenyl Phosphate	3-Phenyl-1-propanol	P(5 HV)		(Makiguchi et al., 2011)
		5.2	1.09	95.0					
ε-caprolactone		21.6	1.07	61.6			P(6HH)		(Makiguchi et al., 2011)
		5.9	1.07	97.1					
Cyclic Diolide		154	1.01	71	Yttrium racemic salen complex	BnOH	P(3HB)	(*R*)	(Tang and Chen, 2018)

generally not achievable by bacterial fermentation. However, more recently, through metabolic engineering it was possible to reorganize the metabolic pathways of the PHA producers, suppressing non-useful pathways and assuring the correct supply of sufficient precursors, so as to achieve very low polydispersity indexes (Tsuge, 2016). Thus, summarizing, mainly due to the large differences in process costs mentioned before, it is clear that the microbiological route is the one of choice for any possible large-scale production.

The situation is quite different when considering the currently available industrial technologies for large scale production, the third factor in our list above, where the microbiological and chemical routes are both in a very favourable position. The technology to carry out, at the industrial scale, fermentation and polymerization exists and it is widespread, and building a new production facility from scratch would require a similar investment, since similar equipment is required in the two cases. Moreover, it would be relatively easy to adapt existing industrial facilities to the bacterial or chemical production of PHA. To achieve the same level of maturity with the enzymatic route, it would require a tremendous effort due to the complexity of these processes.

However, one might ask, if the technology is already there, why industrial units for the production of PHA and the use of PHA-based plastics are not widely spread in our society? The answer is of course in the associated cost that, even for the most convenient case of the microbiological route, is still too high to compete with that of traditional plastics of fossil origin. This justifies the large research effort currently devoted both in industry and in academia directed to the identification of suitable and cheap raw materials, such as waste streams coming from other industrial or civil activities. This requires the definition of suitable microorganisms, possibly properly engineered, and the realisation of a robust reacting system able to produce macromolecules with the required molecular characteristics, in terms of molecular mass and composition distributions. However, this is only one component of a successful industrial technology. More work needs to be done in the direction of process engineering, for example using intensification techniques, including integrated continuous production units.

The case of other industries can be brought forward. For example, the (bio)pharmaceutical industry is now taking the example of oil refineries and other mechanical and chemical industries by investing into fully integrated and continuous production lines, leading to amazing improvement in production costs and product quality (Karst et al., 2018; Pfister et al., 2018). The same can be envisioned for the production of bioplastics. All current PHA producers rely, at least partially, on batch or fed-batch technologies (Koller et al., 2018), and the introduction of such new technologies, possibly in the frame of biorefineries (Dietrich et al., 2017) can significantly boost these natural polymers in the industrial scale scenario.

Acknowledgements

This work has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 812909 CODOBIO, within the Marie Skłodowska-Curie International Training Networks framework.

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