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Bacterial Production of Hydroxyalkanoates (PHA)

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Abstract The dependence of plastic materials is an increasing problem. Although plastics are very useful for humankind many disadvantages derived from the difficulties linked to recycling and disposal are well known. A feasible alternative is the production and use of bioplastics. These compounds have multiple options at the end of his life that can ensure their safety and efficacy of reuse or recovery. For example, raw materials can be returned to the manufacturer for recycling. Bioplastics synthesized through biotechnology include mainly polyhydroxyalkanoates (PHAs), common lipoidic storage materials accumulated by prokaryotes. Some processes for producing PHAs by fermentation using microorganisms have been developed at a different extent. However, biopolymers (PHA) market is under development, and therefore cannot compete with traditional plastics since manufacturing is still more expensive. In this review we have focused on the study of the production processes of bioplastics where bacteria are present, also describing the scaling to industrial level aspects and future trends.

Keywords Bioplastic, Poly-Hydroxyalkanoates, PHA

1. Introduction

The dependence of plastic materials in the XXI Century is such that it comes to be difficult to describe a daily situation in which an object made wholly or partly of plastic is not present. However, compared to the many benefits derived from the use of plastic, rises the serious disadvantage linked to the difficulty of recycling and disposal. This difficulty leads to the accumulation of plastic materials in the biosphere as contaminants [1]. For the production of common plastics, non-renewable fossil reserves are used, which contributes to the depletion of natural energy reserves that the planet has, contributing to the increase in greenhouse gases [2]. These conventional materials, by their nature, are stable from the chemical point of view, which means they remain unchanged for long periods of time in the environment. For this reason it is necessary to develop lines of research to obtain new materials capable of replacing them. Fortunately, there is an alternative, via the production of bioplastics. There is a large family of compounds intracellularly accumulated by bacterial species to serve as energy reserve materials and reducing power. These materials have physico-chemical properties similar to those of conventional petrochemical-based plastics, with the particularity that can be biodegraded by microorganisms to finally produce water and carbon dioxide [3, 4]. They are also some limits regarding their biodegradability: sometimes non-degradable materials are required to produce long-lasting products. Fortunately, its duration is sufficient for most packaging we use and throw away every day so its use contributes to reduce the accumulation of wastes.

Among these new materials stand out poly-hydroxyalkanoates (PHA), plastic biopolymers produced by bacteria [3]. The biotechnological production of such bacterial plastic materials (bioplastics) is one of the promising alternatives being considered to reduce petroleum dependency and achieve a reduction of solid waste and emission of gases that cause the greenhouse effect [5]. This possibility is given by the production of biodegradable polymers, from waste carbon source feedstock. The development of biotechnology has enabled, among numerous other applications, find solutions to two of the most serious problems of modern industry of synthetic polymers: a) the ecological problem that arises from the degradation of recalcitrant synthetic polymers, b) the need to produce novel polymers having predetermined unusual physical properties, and that cannot be obtained by chemical synthesis [5].

Moreover these biopolymers have a wide range of applications in fields such as biomedical, cosmetology and industrial applications. The requirement for these substances could reach more than 1 million tones by the next years [6]. The main properties are biodegradability, biocompatibility, ability to resist exposition to light and high temperature. Biocompatibility is perhaps one of the properties of PHA most currently studied, and as a consequence, there have been a lot of developments in the field of biomedical. Moreover, these bioplastics can be introduced into animal tissues without causing alteration of the biochemical homeostasis [7]. However, at present,

biopolymers (including PHA) market is under development, therefore cannot compete with traditional plastics since manufacturing is still more expensive. The high costs of production and processing is the main obstacle to the expansion of these materials [8-17].

The objective of this review focuses on the study of the production processes of bioplastics where bacteria are present, also describing the scaling to industrial level aspects and future trends.

2. Polyhydroxyalkanoates: bacterial bioplastics

The majority of living beings accumulate different substances in reserve when there is excess of resources in their environment [18, 19]. Some bacteria accumulate reserve substances under certain conditions when the external energy is over the need for the cell to grow and stay. When nutrients become scarce, are used to survive [18]. The component must be used by the cell when the input of external energy is insufficient to maintain the processes of growth, division or cell viability. Under requirement, the compound should be degraded to produce energy assimilable by the cell. From compound backup cell that allows for energy must survive in an unfavorable environment [19].

Polyhydroxyalkanoates (PHA) are some of the most common reserve substances accumulated by prokaryotes [16]. These polymers are accumulated in intracellular granules by numerous species of bacteria, when soil nutrients are essential for growth (such as combined nitrogen, sulfur or phosphates) and there is an excess in carbon source. When external carbon source is exhausted, or if the limiting nutrient is supplied again, the PHA is depolymerized and subsequently metabolized to act as a carbon and energy source. The use of such a polymer is considered a strategy developed by several bacteria to increase their survival in fluctuating environments. They are used as a source of carbon and energy under conditions of scarcity of nutrients, as a source of carbon and energy for entrenchment (*Azotobacter* sp.) and sporulation (*Bacillus* sp.), for the degradation of toxic compounds, and as a source of reducing power (protection of nitrogenase complex nitrogen in fixing bacteria, and also as a constituent of the bacterial cytoplasmic membrane [18].

PHA is stored in granules in the cytoplasm. The number of granules depends on the presence of amphiphilic phasins (structural proteins in the pellet surface) [20]. PHA can be accumulated in levels of up to 90% (dry weight) within the cells, which are also fully biodegradable to CO_2 and water through natural microbiological mineralization. The catalysis occurs in many microorganisms by both intra- and extracellular depolymerases [18-20]. PHA depolymerases have a catalytic triad (serine-histidine- aspartic acid) as the active site. The catalytic serine is embedded in a sequence motif (G-X1-X2-S-G), known as lipase box [21].

The PHA polymers are thermoplastic, can be processed on conventional processing equipment and are, depending on their composition, ductile and more or less elastic. Differ in their properties according to their chemical composition (homo- or copolyester, hydroxy fatty acid content). As mentioned, the compounds are polyesters PHA monomers (R)-3-hydroxy acids, generally linear, in which the carboxyl group forms an ester with the hydroxyl group to the next link monomer [22].

So far there have identified more than 150 hydroxyalkanoic acids: saturated, unsaturated, halogenated and aromatic, which are incorporated into the side chain of PHA and in turn change their physical properties, leading them to be used in new technological applications. This variability depends on the type of substrate, the polymerization specificity and different metabolic pathways involving the formation of monomers supplied. The molecular weight of PHA is about 50-100 kDa, depending on the nature of the polymer [22]. Due to their structural conformation, Short Chain PHA (SCL) and Long Chain PHA (MCL), differ in their thermoplastic characteristics. SCL are classified as thermoplastics, while MCL, having lower crystallinity and lower melting points are recognized as elastomers. MCL have a breaking elongation curves higher than 100%, while the SCL have a lower rupture elongation curves (5%). The characteristics of the environment also affect the production of biopolymers. Several studies have shown the influence of magnetic fields in the yield of microorganisms [22].

2.1. Chemical Structure

In general, PHAs are comprised of several repetitions of the same monomer; found nearly 150 different types of monomers incorporated into the PHAs, which include units hydroxyalkanoates substituted with a wide range of groups: alkyl, aryl, alkenyl, cyano, epoxy, ether, and acid groups [23-25]. As a general rule, the composition and physical properties of PHA produced by microorganisms is dependent of the carbon source used [26]. PHA are linear polymers of (R)-3-hydroxy acids in which the carboxyl group of a monomer forms an ester linkage with the hydroxyl group of the next monomer (Figure 1). According to the length of the carbon chain, PHAs are typically divided into three groups:

- SCL: These are (R)-hydroxyalkanoates whose monomers are composed of 3-5 carbons are thermoplastics with a high degree of crystallization; these crystals form stiff making them less flexible.
- MCL: They consist of monomers (chain length of 6-14 C) and may be aliphatic or aromatic. They possess valuable mechanical properties, are hydrophobic, resilient, have a low degree of crystallization (semicrystalline thermoplastic elastomers) and a low melting temperature, like all PHAs are biodegradable and biocompatible.

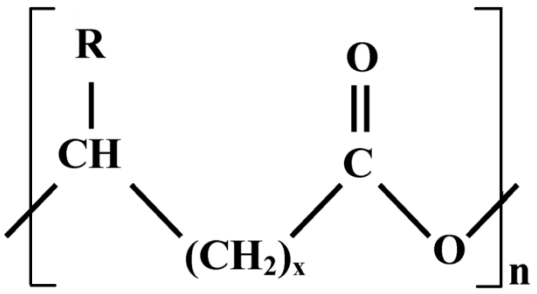

Figure 1. Molecular structure of PHAs. R side chains consist of alkyl groups up to 13 carbon atoms in length, and the number of consecutive CH_2 groups in the polymer backbone (x) ranges from 1 to 4.

- SCL-MCL (Copolymers): Consist of monomers whose chain length is from 4 to 14 C, have a range of physical properties depending on the percentage of the molar composition of the different monomers incorporated into the polymer. Copolymers having a low percentage of monomers SCL are more elastomeric [22]. Singh and Mallick have proposed other categories in spite of this, for *Pseudomonas aeruginosa* cultures: Long-Chain-Length (LCL)-PHAs, SCL-MCL-PHA co-polymer and SCL-LCL-PHA [12-14].

2.2. Synthesis

PHA are biodegradable polymers obtained from a wide range of gram-negative and gram-positive bacteria that accumulate them in the cytoplasm, to act as storage materials such as carbon, energy, and reducing power, that are manufactured by these organisms under unbalanced culture conditions [18, 25]. The most interesting bacteria involved in PHA production are summarized in **Table 1**. An excess of carbon activates the PHA polymerase enzyme encoded by *PhaC*. If starvation occurs, PHA depolymerase encoded by *PhaZ* degrades PHA and R-hydroxyalkanoic acids, to facilitate their use as sources of carbon and energy [18, 27, 28].

Table 1. Production of poly-hydroxyalkanoates in bacteria

Bacteria	PHA composition	Reference
Bacillus cereus	PHB	Ali & Jamil [29]
Bacillus megaterium	PHB	Gouda *et al.* [30]
Bacillus megaterium R11	PHB	Zhang *et al.* [31]
Bacillus megaterium strain JK4h	PHB	Dhangdhariya *et al.* [32]
Bacillus mycoides RLJ B-017	PHB	Borah *et al.* [33]
Comamonas testosteroni	PHB	Thakor *et al.* [34]
Cupriavidus necator H16	PHB	Batcha *et al.* [35]
Cupriavidus necator H16	PHB	Obruca *et al.* [36]
Haloferax mediterranei	PHB	Huang *et al.* [37]
Ralstonia eutropha H16	PHB	Kahar *et al.* [38]
Recombinant *Escherichia coli* arcA	PHB	Nikel *et al.* [39]
*Recombinant *Aeromonas hydrophila* 4AK4	P(3HB-co-3HHx)	Tian *et al.* [40]
*Recombinant *Ralstonia eutropha* H16 strain	P(3HB-co-3HHx)	Kahar *et al.* [38]
*Recombinant *E. coli* DH5α	P(3HB-co-3HHx-co-3HO-co-3HD)	Li *et al.* [41]
Bacillus sp.	P(3HB-co-3HV)	Shamala *et al.* [42]
Bacillus sp. 256	P(3HB-co-3HV)	Kumar *et al.* [43]
Brevibacillus invocatus MTCC 9039	P(3HB-co-3HV)	Sankhla *et al.* [44]
Cupriavidus necator	P(3HB-co-3HV)	García *et al.* [45]
Halomonas campisalis	P(3HB-co-3HV)	Kulkarni *et al.* [46]
Methylobacterium sp. GW2	P(3HB-co-3HV)	Yezza *et al.* [47]
Recombinant *E. coli* XL1	P(3HB-co-3HV)	Yang *et al.* [48]
Serratia ureilytic	P(3HB-co-3HV)	Reddy & Mohan [49]
**Pseudomonas aeruginosa* MTCC 7925	P(3HB-co-3HV-co-3HHDco-3HOD)	Singh & Mallick [14]

3HB: 3-hydroxybutyrate, 3HV: 3-hydroxyvalerate, 3HHx: 3-hydroxyhexanoate, 3HO: 3-hydroxyoctanoate, 3HD: 3-hydroxydecanoate, 3HHD: 3-hydroxyhexadecanoic acid, 3HOD: 3-hydroxyoctadecanoic acid, *SCL-MCL-PHA co-polymer producers, **SCL-LCL-PHA co-polymer producers. Modified from Kumar et al. [12].

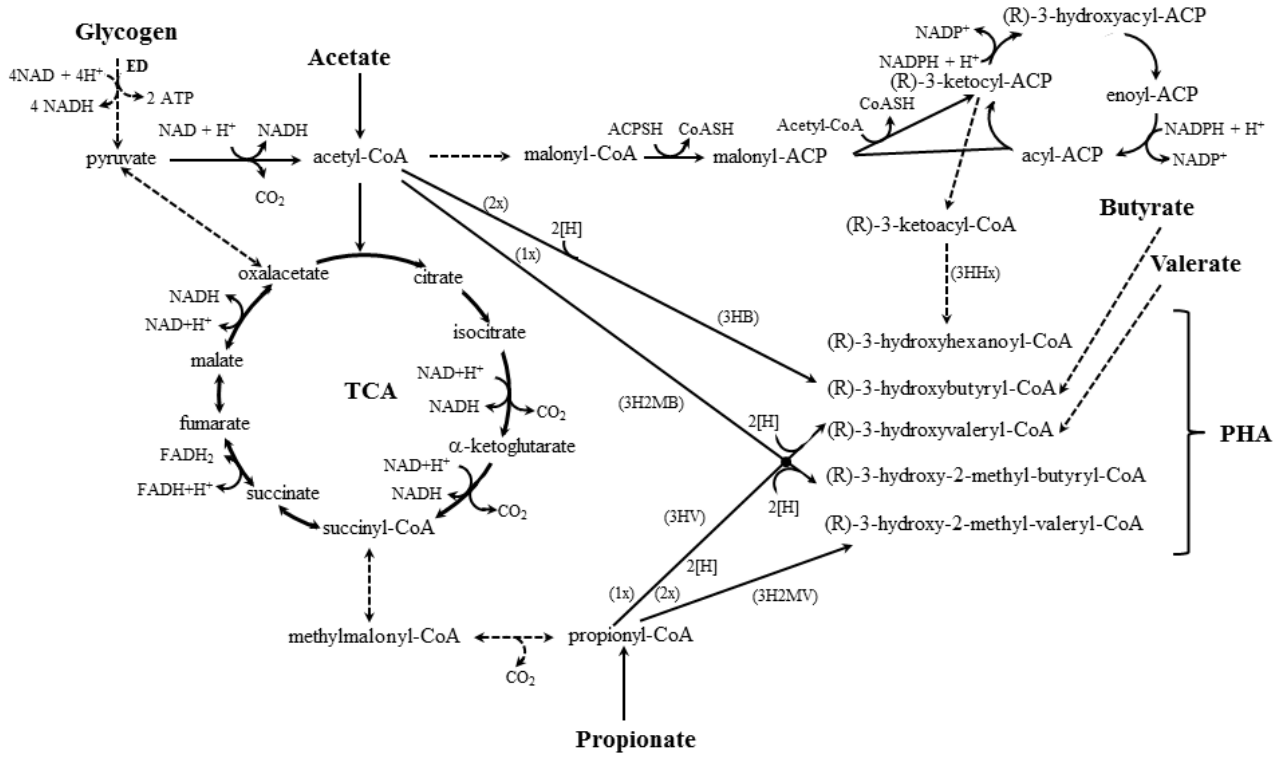

Figure 2. PHA metabolic pathways: ED, Entner-Doudoroff; TCA, tricarboxylic acids

2.2.1. Metabolism and precursors associated to PHA synthesis

Polymer synthesis is related to essential metabolic pathways for the cell (Figure 2), because the necessary precursors may be produced in different metabolic pathways as the tricarboxylic acid cycle (TCA), the β-oxidation of fatty acids and the *de novo* synthesis of fatty acid, involving central metabolites such as acetyl-CoA and cofactors like NADPH [50]. In the genus *Pseudomonas,* MCL biosynthesis is closely related to the central metabolic pathways for cell precursors that can provide for polymer synthesis, as the *de novo* synthesis of fatty acids and β-oxidation fatty acids [28]. The *PhaJ* and *PhaG* genes encoded enzymes to act as a link between these pathways and polymerases, thus providing the monomer required for biosynthesis [51].

2.2.2. ß-oxidation of fatty acids

In the bacterial metabolism, fatty acids are degraded by ß-oxidation by 2 carbon loss in the form of acetyl-CoA. In each cycle the acyl-CoA molecule generated is oxidized to 3-keto-acyl-CoA using (S)-3-hydroxyacyl-CoA as intermediate. The result of this cycle is a fatty acid molecule with n-2 carbon atoms, which can reenter the degradation cycle or be diverted into other pathways [52]. The intermediate (S)-3-hydroxyacyl-CoA cannot be used directly for biosynthesis of PHA, and require the action of the enzyme (R)-enoyl-CoA hydratase (*PhaJ*), which provides (R)-3-hydroxyacyl-CoA thioester which acts as substrate for the PHA polymerase [53]. There are have been cloned and characterized several R-enoyl-CoA hydratases from various PHA-producing bacteria [52].

2.2.3. *De novo* synthesis of fatty acids

Fatty acid synthesis is carried out by a series of enzymatic steps which can involve the activity of enzyme complexes (FASI) or enzymes with a single catalytic domain (FASII). In the first stage a molecule of acetyl-CoA is carboxylated to form malonyl-CoA, therefore this group is transferred to a small carrier molecule of acyl groups (ACP, acyl carrier protein) to form malonyl-ACP. In a successive process, the required residues are added to rend the final fatty acid [26]. The enzyme (R)-3-hydroxyacyl-ACP-CoA transferase (*PhaG*) plays an important role by connecting the de novo synthesis of fatty acid with the MCL synthesis. PhaG catalyzes the conversion of (R)-3-hydroxyacyl-ACP to (R)-3-hydroxyacyl-CoA and contributes to the biosynthesis of MCL from gluconate or other non-carbon sources [53, 54].

2.3. Degradation

There are several routes for metabolization of degradable polymers, basically: a) thermal degradation: by effect of temperature, b) hydrolytic degradation: due to contact with water, c) photodegradation: by sunlight, and d) biodegradation: made by microorganisms. Degradation is faster when low molecular weight polymers are involved. Higher molecular weight polymers require the combination

of photosensitive and hydrolyzable functional groups to achieve an effective environmental degradation [27]. PHAs are degraded by two major pathways, one intracellular and other extracellular by PHA-hydrolases and PHA-depolymerases [26, 55]. However, the degradation time of a piece of PHA ranges from a few months to years depending on the plastic composition and environmental conditions [56].

3. Microbial Production Processes

To develop a process for producing PHAs by fermentation using microorganisms is necessary to optimize the performance and the purification steps of the polymer, and fundamentally to reduce the cost of the substrates used for their production [55]. Nowadays, there are several processes developed for the production of PHAs by fermentation from inexpensive substrates: in Brazil is produced from molasses and in the United States and Korea from various substrates of plant origin [57]. Research on PHA in recent years aimed at reducing production costs and increase productivity using various strategies. To achieve these objectives is essential to optimize the fermentation process. Recent work has demonstrated the importance of studying the overall response to stress in the development of these processes, noting the need for both basic research and applied to the design of biotechnological processes involving fermentation steps [8, 57]. Bacterial synthesis of PHAs has been described in over 300 species. However, before the selection of microorganisms suitable for the industrial production of PHAs various factors should be considered: a) the ability of the cell to grow on not very expensive carbon sources, the speed or rate of growth, the rate of synthesis of the polymer and the maximum possible polymer accumulated by the cell according to the available substrate. Several studies have proposed equations to predict PHA yield in function of the carbon used, which can be quite useful for preliminary calculations [57, 58].

Successful approaches to produce PHA have been proposed for several microorganisms, such as *Ralstonia eutropha* [6], *Rhodobacter* [59, 60], *Azospirillum rubrum* [61], *Azotobacter* [62], *Methylocystis* [63], *Alcaligenes* [64], *Leptothrix* [65], *Pseudomonas* [19, 51, 52, 55, 66], *Baggiatoa* [63], *Rhizobium* [63] *Enterobacter* [67] or *Bacillus* [68]. The isolation of each of the microorganisms used for production of PHAs is directly related if it is native or recombinant. In the first case their identification and selection must be made by microbiological techniques (biochemical tests, morphological), in the second case because these microorganisms have been genetically modified by inserting usually plasmids, identification and selection is done using specific techniques depending on the properties of these plasmids (antibiograms or degradation or specified substrates) [58].

5. Conclusions

The problems of traditional plastics waste is a persistent problem. The vast majority of plastic products that are not recycled are discarded in landfills, while others are deposited by wind in non-traditional landfills, like the famous Great Pacific garbage patch. Bioplastics have multiple options at the end of his life that can ensure their safety and efficacy of reuse or recovery. For example, raw materials can be returned to the manufacturer for recycling.

To develop a process for producing PHAs by fermentation using microorganisms is necessary to optimize the performance and ease of purification of the polymer, and fundamentally reduce the cost of the substrates used for their production.

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