

Microbial Production of Bioplastics: An Eco-friendly Alternative

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ABSTRACT

Accumulation of non-biodegradable plastic has shown adverse impacts on the environment and calls for a dire need for a sustainable alternative. Various microbial strains can produce bioplastics in the form of Polyhydroxyalkanoates (PHAs) as energy reserves. Many bacteria, fungi and microalgae have been studied to produce such biopolymers. PHAs are biodegradable and meet the basic requirements of life cycle environmental impact or life cycle assessments for proper disposal. They are also biocompatible and renewable. They have high Elastic modulus, Tensile modulus, melting temperature, and crystallinity with many other properties similar to synthetic plastics currently in use, making them a more reliable and sustainable substitute. Bioplastics produced from PHAs have found a myriad of applications in medicine, pharmaceuticals, agriculture and the packaging industry. This review emphasizes the structure of PHAs, their biosynthesis and relevant microbial strains employed, including genetically engineered strains, microbes from extreme niches and mixed microbial cultures. It focuses on using cheap and sustainable carbon feedstocks, including agricultural residues, lignocellulosic biomass and crude glycerol, on making the production of PHAs cleaner and commercially feasible. Industrially scaled production using different fermentation strategies, downstream processing and purification, along with the wide range of applications of PHAs, is also discussed.

Key words: *Bioplastics, Microbial production, Biopolymers, Polyhydroxyalkanoates, Biodegradable*

Introduction

Plastic is one of the most significant innovations in the history of mankind. It can be defined as a non-biodegradable synthetic polymer that has applications in various fields and can be manufactured into multiple important goods and products. Although, in spite of its beneficial properties, there exists a widespread issue of its deleterious impacts (Getachew and Woldesenbet, 2016). During the last few decades, the utilization of synthetic polymers has increased rapidly, leading to their piling up over time and polluting the planet. This calls for a des-

perate need to find a bio-sustainable alternative to salvage the environment. In view of the severely harmful impacts of plastics on the environment as well as public health, it is essential to create biopolymers that could be environment friendly, sustainable and applicable to the production of all necessary goods that are being produced with synthetic polymers at present (Luengo *et al.*, 2003).

Bioplastics are natural products that can be synthesized by various organisms through metabolic pathways (Angelini *et al.*, 2015). Such biomaterials, owing to their eco-friendly properties, are considered the best alternative to conventional plastic ma-

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materials that are currently being commercially produced. The renewability and biodegradability of bioplastics have made them an important part of global sustainability. Biological recycling of bioplastics has also given new support to waste reduction and management. Certain microorganisms possess the ability to synthesize microbial polyesters such as polyhydroxyalkanoates (PHA) which can be used to produce bioplastics. Polyhydroxyalkanoates are natural polymers that are biodegradable and biocompatible, meaning that they do not exhibit a toxic response in the host (Steinbüchel and Fächtenbusch, 1998). They are synthesized by a wide variety of bacteria, fungi and algae as a response to insufficient supply of nutrients such as nitrogen, phosphorus and other compounds like RNA and enzymes that may be essential for growth, as well as excess supply of carbon sources (Ren *et al.*, 2009). In such unbalanced conditions, PHAs act as energy storage units in the microbial cells (Sedlacek *et al.*, 2019) and are present in the form of inclusions or granules (>0.5 μm in size) inside the cytoplasm (Sudesh *et al.*, 2000). The main role of PHAs is to allow microbes to adapt to harsh and stressful environmental conditions caused by the scarcity of nutrients.

Polyhydroxyalkanoates

PHAs are an extremely varied class of polymers having over a hundred different kinds of monomers (Jain *et al.*, 2010). Varying compositions of monomers give rise to diverse forms of PHAs that exhibit different physicochemical properties, based on which they can be categorized for application in various industries such as medical devices, pharmaceuticals, biofuels, and packaging, bottle manufacturing, etc. (Kalia *et al.*, 2019). Poly-3-hydroxybutyrate (P3HB), Poly-4-hydroxybutyrate (P4HB), Polyhydroxyvalerate (PHV) and Poly(hydroxybutyrate-co-hydroxy valerate) (PHBV) are a few of the PHAs that can be produced by microorganisms (Yamaguchi *et al.*, 2019). The most commonly studied PHA is Poly-3-hydroxybutyrate (also abbreviated as PHB), which is formed due to the substitution of the functional group by hydroxybutyrate. PHB is thermo resistant and easily moldable, making it a suitable replacement for petroleum-derived plastics. Due to its biocompatibility, it also has wide applications in fields and products such as medical devices, surgical implants, drug delivery systems and plastic sur-

gery. Amongst all the biodegradable plastics that are currently being industrially produced, PHB is the only one that is a hundred per cent biodegradable.

The production of different types of PHAs depends upon different microbial strains. Recent studies have shown that there are now about 300 known species of microorganisms, including several bacteria, fungi and algae, that are capable of synthesizing various forms of polyhydroxyalkanoates, of which most commonly studied are bacteria (Zinn *et al.*, 2001). These microbes use several different substrates that act as a carbon source for the production of PHA, including agricultural waste material, industrial wastewater, fermented molasses, oil mill effluents, lignocellulosic biomass, residues from food industries, forestry, municipalities, etc. (Bhatia *et al.*, 2021). However, the cost of these feedstocks used as carbon sources amounts to approximately 50% of the total cost of the resulting bioplastic that is produced, making it a major drawback when compared to the cost of conventionally used non-biodegradable plastics derived from petroleum.

Synthesis of Polyhydroxyalkanoates (Phas) in Bacteria

A large number of bacterial strains are capable of synthesizing PHAs. During stressful environmental conditions with insufficient nutrients available for growth along with excessive amounts of carbon, bacteria begin to accumulate PHAs to store energy. This occurs when there is limited availability of nitrogen, phosphorus and, in some cases, oxygen (Anderson *et al.*, 1995). Because of insolubility in water, the PHAs are accumulated in the form of granular bodies inside the cytoplasm of bacterial cells. The osmotic state of the cell is maintained through polymerization of the soluble intermediates and the formation of insoluble compounds. This prevents the polymerized molecules from leaking outside the cell and, as a result, maintains the energy reserves in harsh conditions (Peters and Rehm, 2005).

Genes and enzymes involved in biosynthesis

The granular surface is coated with phospholipids and proteins. Phasins are a class of proteins that are chiefly found in the interface of the PHA granule. Phasins affect the quantity as well as the size of the PHA granules (Pötter and Steinbüchel, 2006). The major enzymes involved in the synthesis of PHAs are PhaA, PhaB and PhaC. In addition to these cru-

cial enzymes, PhaP is also involved and is responsible for the accumulation of PHAs and the morphological integrity of the granules.

Polyhydroxybutyrate was the first PHA found to be produced by bacteria, and hence, it is the most researched to this date. Numerous bacterial strains are known to be capable of converting sugars and fatty acids into PHB via three different metabolic pathways that involve acetyl-CoA as the intermediary species (Quillaguamán *et al.*, 2010). The biosynthesis of PHAs is initiated with the combination of two molecules of acetyl-CoA by the action of 3-ketothiolase (PhaA), leading to the formation of acetoacetyl-CoA. Acetoacetyl-CoA reductase (PhaB) is an NADH dependent enzyme that reduces acetoacetyl-CoA to 3-hydroxybutyryl-CoA. At last, the polymerization of 3-hydroxybutyryl into polyhydroxybutyrate (PHB) by PHA synthase (PhaC) takes place, releasing coenzyme-A (Tsuge *et al.*, 2005). The 3-ketothiolase enzyme is inhibited by free coenzyme-A from the Krebs cycle under normal growth conditions. However, under nutrient-limited conditions with abundant carbon supply, the entrance of acetyl-CoA into the Krebs cycle is constrained, and the excess acetyl-CoA is directed to PHB biosynthesis (Ratledge and Kristiansen, 2001).

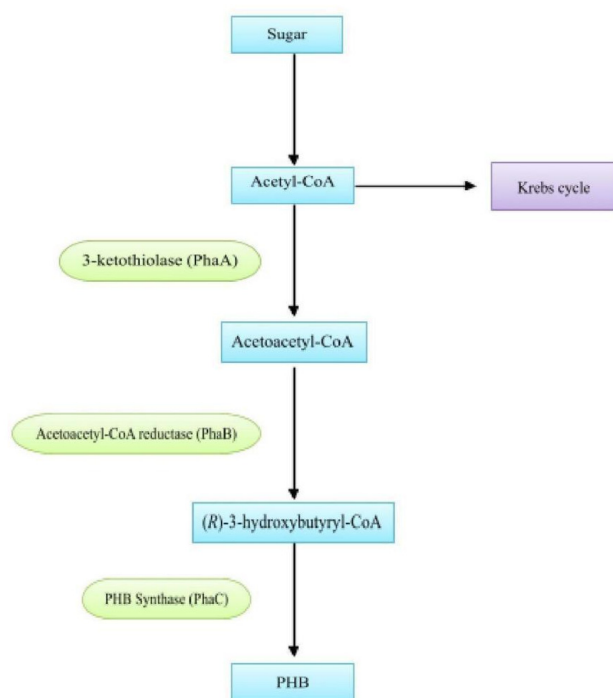


Fig. 1. Metabolic pathway of PHB synthesis

Most suitable microorganisms for the production of PHAs

Production of PHAs in microorganisms was first discovered by a French scientist named Maurice Lemoigne in 1926 when he observed the formation of PHB in the bacterial species *Bacillus megaterium* (Koller, 2019). He demonstrated that a transparent plastic-like material could be made by utilizing the extracted PHAs. A large variety of bacteria, fungi as well as microalgae possess the ability to produce polyesters like PHAs. In the last decade, hundreds of bacteria that can accumulate PHAs have been identified, some of which are found to be more competent than others as they can produce PHAs more efficiently and at a more significant rate (Chodak, 2008).

Some fungal sources of PHA include *Aspergillus fumigatus*, *Saccharomyces cerevisiae*, *Arxulaadeninivorans* and *Pichia pastoris*. Various species of microalgae such as *Aulosirafertilissima*, *Botryococcusbraunii*, *Spirulina platensis*, *Nostoc muscorum* and *Chlorella minutissima* are also capable of PHA synthesis (Muneer *et al.*, 2020).

Use of mixed microbial cultures (MMC)

According to recently conducted studies, the use of mixed microbial cultures (MMC) is more advantageous than pure cultures for synthesizing PHAs. Monocultural production of PHAs has been observed to provide lesser yield and slower accumulation than when mixed cultures are used (Ashby *et al.*, 2005).

The most common source of MMC is activated sludge. Mixed cultures possess the ability to quickly convert complex substrates from sources such as industrial wastewater into PHAs. Usually, the most dominant microorganisms in the MMC involved in the conversion of substrates from this source are found to be Proteobacteria and Acidobacteria. Research has shown that such mixtures can also tolerate toxic phenolic compounds (Zhang *et al.*, 2018), do not exhibit a high demand for sterility (Reis *et al.*, 2011) and can completely utilize most of the naturally available, inexpensive and complex substrates, providing high levels of PHA accumulation.

Genetically engineered strains

In recent years, genetic engineering has made headway and has delivered notable improvement in the PHA synthesizing processes. Genetic manipulation

of microbes was first seen in 1988 when Dennis *et al.* managed to clone all the genes of *Ralstonia eutropha* involving the three 'phasins' responsible for the synthesis of PHB. These genes were then introduced into *Escherichia coli*; as a result, it acquired the ability to accumulate PHB in high yield percentages. In the following years, various recombinant strains of *E. coli* were engineered, some of which have been observed to synthesize HV copolymers. Moreover, maximum utilization of cheap and renewable carbon sources like soybean oil, palm oil, and whey (Ahn *et al.*, 2001) by recombinant *E. coli* strains has been observed, producing 3HHx and 3HO monomers.

Essential growth conditions and use of renewable substrates

As mentioned earlier, the accumulation of PHAs in microbes takes place in scarce conditions where essential nutrients like nitrogen, phosphorus and oxygen are limited while carbon is present in excess. In order to produce PHAs industrially, nitrogen supply can be limited simply by excluding ammonia from the growth media or providing extremely low concentrations of nitrogen supplements such as (NH)SO and NHCl. It is essential to maintain a balanced carbon to nitrogen ratio in the growth media to achieve optimum PHA production of PHAs (Raza *et al.*, 2019). Studies have shown that the effects of nitrogen and carbon on the production of PHAs vary according to the microbial strain. The ratio of carbon to nitrogen also affects the amount of PHA produced in microbes.

The most important component in the production of PHAs is carbon because it serves as the energy source for all metabolic pathways in microbes. All PHA producing microbes are dependent on naturally available carbon sources such as glucose, sucrose and lactose (Hawas *et al.*, 2016). Currently, the industrial production of PHAs is using carbon sources that are made commercially, because of which the total cost of the product is exceptionally

high, and the production rate is low. Recent studies have explored various natural feedstocks serving as a rich carbon source, including wheat, molasses (Favaro *et al.*, 2019), rice (Peña *et al.*, 2014), cheese whey, agricultural wastes, oil wastes, lignocellulose, vinasse, fermented crude glycerol (Li and Wilkins, 2020) and many others that may lower the production costs and increase the rate of production. The use of these natural feedstocks is not only inexpensive but also sustainable. It aims at saving energy and non-renewable substrate sources. The biodegradability of bioplastics ensures continuous recycling of such natural substrate sources.

Production Strategies in Industries

With the growing interest of industries in bioplastics, research interest in the development of creative and efficient strategies has also notably increased in the last few decades. The main objective of industries producing bioplastics is to adopt strategies that have maximum productivity by applying environment-friendly processes and minimum cost, ensuring sustainable development. The production cost of PHAs depends not only on the inexpensiveness of the carbon source being utilized but also on the production strategy in place. Numerous advances have recently been made in overcoming the limitations that arise in large scale production through conventional industrial methods. For example, the use of recombinant microbial strains that synthesize PHA from cheap carbon sources is being widely studied. There is continuous research on the improvement of cleaner fermentation processes for bioplastic production. Moreover, different approaches such as enzymatic synthesis in bioreactors are being explored with the aim to scale up the process to an industrial level.

Fermentation strategies used in bioplastic manufacturing industries

Production of PHAs on an industrial scale requires controlled conditions that can be provided by large

Table 1. Examples of bacterial strains and their produced PHA types.

Bacterial strain	Type of PHA produced	Reference
<i>Alcaligenes latus</i>	(3HB-co-3HHx)	(Chodak, 2008)
<i>Azotobacter vinelandii</i>	P(H3B)	(Spiekermann <i>et al.</i> , 1999)
<i>Cupriavidus necator</i>	P(3HB-co-3HV)	(Amaro <i>et al.</i> , 2019)
<i>Escherichia coli</i>	mcl-PHA	(Sohn <i>et al.</i> , 2020)
<i>Methanomonasp.</i>	P(3HB)	(Braunegg <i>et al.</i> , 2002)

fermenters operating in different modes. Optimum results depend upon skilled adjustments in both feeding strategy and the mode of operation of the fermentor. The conditions of the fermentation process are determined on the basis of the microbial strain being used. Usually, the operating temperature is kept in the range of 30 to 37°C, combined with low humidity and low dissolved oxygen due to low, stirring speed. The pH levels are sometimes left unregulated, and other times they can be regulated with correspondence to the addition of a substrate. Various types of fermentation strategies have been developed and are still under intensive research for improving the efficiency of industrial production of bioplastics.

Batch mode

Traditionally, batch fermentation has been the preferred setup for the production of bioplastics in industries. However, this mode is known to provide limited productivity because most carbon sources are converted towards biomass and yield a very low volume of PHAs (as demonstrated in Table 2), and it is proven to be economically infeasible. On the other hand, fed-batch fermentation has been stated to boost the production volume because of the addition of substrate as soon as its concentration is reduced to a certain level.

Fed-batch mode

When operating a fed-batch fermentation mode, the substrate is introduced into the system in pulses when the pulses fall below a certain level, while the culture is not removed (Koller, 2018). During PHA production via fed-batch mode, nitrogen and carbon

sources can be re-fed in repeated intervals based on biomass consumption until a desired concentration of biomass with low PHA content is obtained. In the growth phase, nitrogen feeding can be directly related to the change in pH as biomass formation is also proportional to the decrease in pH levels (Ahn *et al.*, 2000). Ammonia is used as a pH regulating agent, keeping both nitrogen levels and pH at a constant value without extensive monitoring of the nitrogen source and its input levels (Ahn *et al.*, 2001). NaOH is used as a corrective pH regulator and is used to replace ammonia in order to start the transition from the growth phase into the PHA producing phase (da Cruz Pradella *et al.*, 2012). Supply of Carbon is performed via the addition of substrate pulses till the completion of the process, which is indicated by a slowdown of PHA production rates.

Feast and famine strategy for enhanced production

The feast-famine method is a popularly adopted strategy in the industrial production of PHAs. Wastewater can be used for PHA production via utilization by mixed microbial cultures, and the feast-famine strategy can significantly enrich the PHA synthesizing microbes (Morgan-Sagastume *et al.*, 2015). This approach involves feeding a substrate in intervals, wherein one phase, the carbon substrate and oxygen are abundantly supplied, leading to intracellular accumulation of PHAs (Fradinho *et al.*, 2016). This is referred to as the 'feast' phase, followed by the halting of substrate and oxygen supply, known as the 'famine' phase.

Downstream processing

Downstream processing for recovery of PHA from

Table 2. Strains for PHA production by batch fermentation mode.

Strain	Carbon source	PHA fractions concentration (g/g)	Reference
<i>Chelatococcusdaeguensis</i> TAD1	Glycerol	0.81	Cui <i>et al.</i> , 2015)
<i>Cupriavidusnecator</i> H16	Jatropha oil	0.90	(Ng <i>et al.</i> , 2011)
<i>Halomonascampisalis</i>	Maltose	0.79	(Kulkarni <i>et al.</i> , 2010)
<i>Bacillus firmus</i> NI 0830	Rice straw hydrolysate	0.89	(Sindhu <i>et al.</i> , 2013)

Table 3. Strains for PHA production by fed-batch mode.

Strain	Carbon source	Type of PHA produced	Reference
<i>Cupriavidusnecator</i>	Soybean oil	scl-PHA	(da Cruz Pradella <i>et al.</i> , 2012)
<i>Haloferaxmediterranei</i>	Cyanophycin	scl-PHA	(Hermann-Krauss <i>et al.</i> , 2013)
<i>Pseudomonas chlororaphis</i>	Animal waste	mcl-PHA	(Muhr <i>et al.</i> , 2013)
<i>Escherichia coli</i> CGSC 4401	Bovine whey solution	PHB	(Ahn <i>et al.</i> , 2000)

the biomass produced during fermentation can be quite expensive, accounting for at least half of the total production cost. This is because of the relatively low amount of the polymer being produced as per the PHA being accumulated intracellularly. A handful of research on the recovery and purification of PHA has recently been published, with the ultimate goal of developing competitive procedures for industrial exploitation.

Separating the biomass, i.e. the cells that contain PHA, from the broth is the first step following fermentation. The most commonly used procedures are centrifugation, filtration and sedimentation. The permeability of the microbial cells can be increased by pre-treating the biomass. This can be achieved via heat treatment, the addition of salts, liquid nitrogen or subjecting the biomass to cold temperatures. In accordance with the nature and composition of the PHAs, the biomass undergoes further recovery processes.

Digestion of cell mass

The primary goal of cell mass digestion is to dissolve the non-PHA cell mass while keeping the PHA granules intact. This can be accomplished via chemicals such as acid and alkalis or via enzymatic treatment. For the last few years, industries have adopted the method of using strong oxidizing reagents like sodium hypochlorite and sodium hydroxide for the dissolution of non-PHA cell mass. However, it is vital to effectively regulate the concentration of oxidizing reagents being used as high concentrations will dissolve the accumulated PHA along with the non-PHA biomass, resulting in limited recovery as well as degradation of PHA.

Direct extraction of PHA from biomass

The most frequently used recovery method in the production of PHAs is solvent extraction. In this

approach, the PHA containing biomass is immersed in a suitable solvent or mixture of solvents. This is followed by the recovery of PHAs by adding precipitant solvent, which causes the PHAs to crystallize. Halogen containing solvents like chloroform and methylene chloride are commonly used in this method. However, such solvents are known to be toxic to the environment when used on a large scale. In this regard, non-halogenic solvents like methanol and propanol are preferred for the extraction of PHAs.

Purification

Depending on the extraction procedure employed, the type of impurities that remain in the recovered biopolymer varies. With the use of non-polar solvents, lipids and pigments are usually co-extracted, whereas proteins are most often separated after the cell mass has been chemically degraded in an aqueous solution.

In the case of the polymer having applications in medicine, the purification process must meet stringent specifications. Contaminants having a biological activity that might cause immunological responses must be minimized to levels that conform with the US pharmacopeia standards. For instance, the outer membrane of gram-negative bacteria contains lipopolysaccharides that function as endotoxins and cause undesirable effects when they interact with blood. Therefore, they are a major concern, and medical-grade PHA must be thoroughly purified to eliminate them. Hypochlorite, inorganic peroxides and organic peroxides (mostly hydrogen peroxide and benzoyl peroxide) can also be employed to further decrease the endotoxin concentrations.

Conclusion

The current global plastic consumption patterns en-

Table 4. Various reagents and their working for extraction of PHA from biomass.

Type of Reagent	Examples	Mechanism of action
Acids	Sulphuric acid	High pH disrupts cellular material and liberates intracellular PHA.
Surfactants	Sodium dodecyl sulphate, Palmitoyl carnitine, Linear alkylbenzene sulphonic acid	Enters lipid membrane and increases volume of cell envelope until it bursts. Formation of micelles of phospholipids and surfactants releases PHA.
Enzymes	Alcalase, Lecitase, Trypsin, Bromelain, lysozyme	Destabilizes membrane, hydrolyses and digests non-PHA cell mass.
Supercritical fluids	Supercritical carbon dioxide (sCO ₂)	Diffuses through the cell membrane and causes complete disruption of the cell.

courage the production of biodegradable plastics, aiming to replace the harmful synthetic plastic compounds that are currently being used in order to salvage the environment and prevent polluting our planet even further. The non-biodegradability of conventionally produced petroleum-based plastics that has led to dangerous amounts of plastic accumulation over the years now has worldwide ramifications that include ozone depletion, carcinogenicity, global warming, and environmental toxicity. Polyhydroxyalkanoates have attracted significant attention due to their valuable characteristics, such as biodegradability and biocompatibility. Their ability to be biologically recycled has made waste management significantly more manageable. Microbial synthesis of bioplastics in the form of PHAs is a potential approach. Diverse microbial species produce different biopolymers from a wide range of carbon feedstocks. However, the large-scale production of bioplastics is still quite challenging due to the high costs of carbon feedstocks. Researchers have managed to find new gateways by focusing on the use of cheap and naturally available carbon feedstocks, reducing the production costs and making it more sustainable.

Additional steps can be taken to improve the industrial production of bioplastics. Genetically engineered microbial strains are being studied for improved production of PHAs on a larger scale. Mixed microbial cultures can be improved through biotechnological techniques, adjustments in operation strategies and culture conditions. It is also notable that different combinations of microbes and substrates demand different fermentation techniques, bioreactor facilities and feeding strategies. Combinations of various fermentation strategies can be studied more extensively to enhance both the quality and yield of the product while promoting sustainability.

In conclusion, if the constraints that limit its economically feasible production at large scales are overcome, bioplastics have a promising future with the potential to not only revolutionize material science, medical and industrial sectors but also to replace synthetic plastics in their entirety, ensuring environmental sustainability.

Conflict of interests: All the authors declare that they have no conflict of interests.

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