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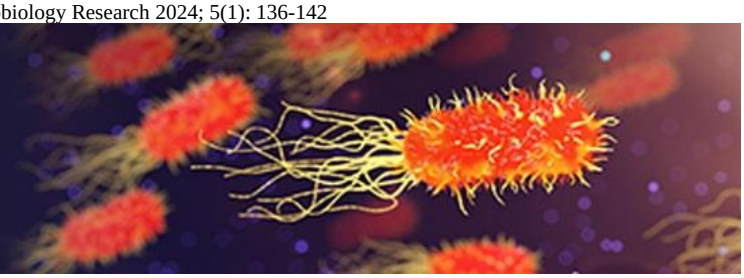

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A comprehensive review on production of polyhydroxybutyrate (PHB)

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Abstract
Polyhydroxybutyrate (PHB) stands out as a biocompatible and biodegradable thermoplastic polymer produced by specific bacterial strains. This comprehensive review explores the production of PHB, highlighting its potential applications and challenges. Various microbial sources capable of synthesizing PHB, including bacteria, cyanobacteria, and yeasts, are discussed alongside their optimal growth conditions and substrate preferences. Additionally, genetic engineering approaches for enhancing PHB production in bacteria, particularly *Escherichia coli*, are examined. Fermentation methods for PHB production, such as batch culture and fed-batch systems, are evaluated, emphasizing the need for precise environmental control. Despite the promising outlook for bioplastics from bacterial sources, challenges remain in strain selection, genetic manipulation, and scaling up production processes. Addressing these challenges will be crucial for realizing the full potential of PHB as a sustainable alternative to traditional plastics.

Keywords: Environmental pollution, biopolymer, polyhydroxybutyrate, microbes, fermentation methods

Introduction
The next generation of materials, goods, and processes has been heavily influenced by sustainability, environmental concerns, and green chemistry in recent years. The development of biodegradable polymers from renewable resources has been fueled by the persistence of plastics in the environment, the depletion of petroleum resources, the scarcity of landfill space, and the worries about the release of harmful gases during burning [1]. Poly-3-hydroxybutyrate (PHB) is polyester that is produced in specific bacterial cells. It comes in the polyhydroxyalkanoates (PHAs) family. It combines with 3-hydroxybutyrate monomers in PHB one monomer forms an ester bond which has a carboxyl group and binds with a neighbor monomer with a hydroxyl group [2, 3]. Polyhydroxybutyrate (PHB) constitutes a biodegradable and biocompatible thermoplastic polymer amenable to fabrication into a spectrum of consumer goods, encompassing plastics, films, and fibers [4]. At present, over 300 bacterial species are recognized for their capacity to synthesize polyhydroxybutyrate (PHB); nonetheless, only a select few among them have been effectively harnessed on an industrial scale for the production of this polymer [5]. Polyhydroxybutyrate (PHB) can be synthesized and stored by various cyanobacterial species, including Spirulina sp. (*Arthrospira* sp.), *Synechococcus* sp., and *Synechocystis* sp. [6-8]. When PHB is disposed of in the environment, it undergoes biodegradation to produce carbon dioxide (CO2) and water [9, 10]. Additionally, when incorporated into blends, PHB can achieve disintegration levels surpassing 90% within 28 days [11].
Typically, the synthesis of polyhydroxyalkanoates (PHAs) occurs under conditions where growth is constrained due to unfavorable environmental factors and imbalances in nutrient availability. This entails an ample presence of carbon sources in the surrounding medium, juxtaposed with restricted supplies of other essential growth elements such as nitrogen, phosphorus, dissolved oxygen, or specific micro-components like sulfur or essential metals necessary for cellular metabolism. These conditions prompt the microorganisms to engage in PHA synthesis, utilizing carbon and energy reserves within the cells [12].

Biodegradable Plastic Polymers
There are three types of biodegradable plastic based on biodegradation: 1. Completely biodegradable, 2. Photo-biodegradable, and 3. Semi-biodegradable. Completely biodegradable plastic includes plastic which has intrinsic biodegradable characteristics.

Specifically, photo-biodegradable polymers feature additives incorporating polymer chain sensitivity responsive to light. The deterioration of these plastics begins upon exposure to ultraviolet radiation, leading to the possibility of eventual complete biodegradation. Semi-biodegradable blends amalgamate biodegradable polymers like starch with nonbiodegradable polymers. Consequently, microorganisms can target and initiate the biodegradation of starch within the blend; however, the non-starch component of the polymer remains resistant to biodegradation [51].

Completely biodegradable polymers are grouped into four categories

1. **Agro-polymers:** These are typically acquired through the fractionation of biomass derived from agricultural products and are further categorized into polysaccharides, proteins, and lipids.
2. **Microbial biopolymers:** These polymers are synthesized by microorganisms utilizing renewable sources, exemplified by polyhydroxyalkanoates (PHAs) produced through microbial synthesis.
3. **Chemically semi-synthesized polymers:** These are derived from monomers obtained via biotechnological processes, such as poly lactic acid (PLA), which is synthesized by polymerizing lactic acid generated through microbial fermentation.
4. Polymers sourced from non-renewable origins, specifically fossil fuels, are synthesized through chemical processes. An example of such polymers is aromatic copolyesters like PBAT [52].

PHA

The presence of bright, lipophilic granules within Beijeirinckia cells was initially documented in 1888, but it wasn't until 1923 that Maurice Lemoigne, using *Bacillus megaterium*, identified the first compound—P(3HB)—as a probable storage material. Throughout the subsequent thirty years, research on P(3HB) mainly focused on method development for the detection and quantification of bioplastic content in cells, as well as exploration of culture conditions conducive to synthesis and degradation by Bacillus. In 1958, Wilkinson Macer observed an increase in storage granules of B. megaterium when glucose and nitrogen proportions in the medium were elevated, suggesting that these compounds served as potential carbon and energy sources for the microorganism under investigation [53].

In the 1990s, the technology for producing PHAs, which are biodegradable and biocompatible plastics, was introduced in Brazil through collaborative efforts between the Institute for Research and Technology (IPT) and the University of Sao Paulo. Researchers utilized carbohydrates as the primary raw material and explored strategies to leverage indigenous microorganisms sourced from Brazilian soil. Brazil's advantageous position in terms of low-cost sugar production, coupled with its surplus energy from sugar and alcohol, rendered it an optimal location for integrated polymer production. In September 2000, PHB Industrial inaugurated production facilities in Sao Paulo, Brazil, becoming the first company to produce and commercialize biodegradable plastic, specifically P(3HB), marketed under the brand name Biocycle [54]. The industry functioned at a pilot scale until 2015 and even exported products to Japan; however, the industrial plant is currently inactive. PHAs constitute a category of polyesters sourced from various microorganisms. These microorganisms accumulate as granules within cells, serving as an intracellular carbon reserve for energy purposes (2). The accumulation of these granules can surpass 80% [55]. These polymers have garnered considerable interest among researchers due to their thermoplastic, biodegradable, and elastomeric properties, all of which can be derived from renewable sources [55, 56].

A significant characteristic of PHA is its biodegradability. In natural settings, numerous microorganisms possess the capability to break down PHA using hydrolase enzymes and internally produced PHA A significant characteristic of PHA is its biodegradability. In natural settings, numerous microorganisms possess the capability to break down PHA using hydrolase enzymes and internally produced PHA depolymerase, thus enabling PHA to serve as a carbon source. However, these enzymes are unable to degrade the polymer externally, meaning once it's removed from the cell, potentially due to the transition from a crystalline to an amorphous state [57]. Many microorganisms present in the soil, marine environments, and lakes possess extracellular PHA depolymerase enzymes, which can degrade the polymer after it has been released from the cell, thereby facilitating its utilization as a carbon source. Its utilization as a carbon source [58].

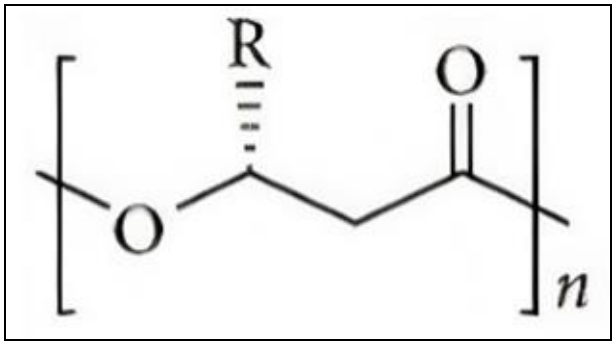

Fig 1: Chemical Structure of polyhydroxyalkanoate (PHA) molecules [32]

PHB

PHB is the most extensively researched and characterized PHA to date. It is a natural polymer and belongs to the category of biodegradable aliphatic homopolymers, consisting of monomers composed of four carbon atoms [59]. It exhibits solubility in certain organic solvents while remaining insoluble in water [56]. Its chemical formula, (C4H6O2)n, translates to a composition of 55.81% carbon, 7.03% hydrogen, and 37.16% oxygen by weight [60]. Industrial interest in the utilization of P(3HB) began to arise in the 1960s, coinciding with the initial documentation of its thermoplastic characteristics [61]. The synthesis of P(3HB) was initially achieved in 1971 through the polymerization of a racemic mixture of β-butyrolactone using a catalyst system comprising trimethylaluminum and water. This process yielded a stereoregular polymer that exhibited partial optical inactivity. Additionally, further research demonstrated the biotechnological production of P(3HB) from bacteria, resulting in a polymer with a low molar mass (1x104 Da) and crystallinity (29%) [60, 62].

Due to its notable thermoplastic characteristics, untreated P(3HB) demonstrates resistance across a broad temperature spectrum, spanning from 30 °C to 120 °C, and can be

manipulated similarly to traditional thermoplastics during processing. Moreover, P(3HB) is biocompatible and non-toxic, with its degradation yielding 3-hydroxybutyric acid, a natural component of human blood even at elevated concentrations. Consequently, P(3HB) finds application in products intended for contact with human or animal tissue as well as for human consumption [63].

Biosynthesis pathway of PHB

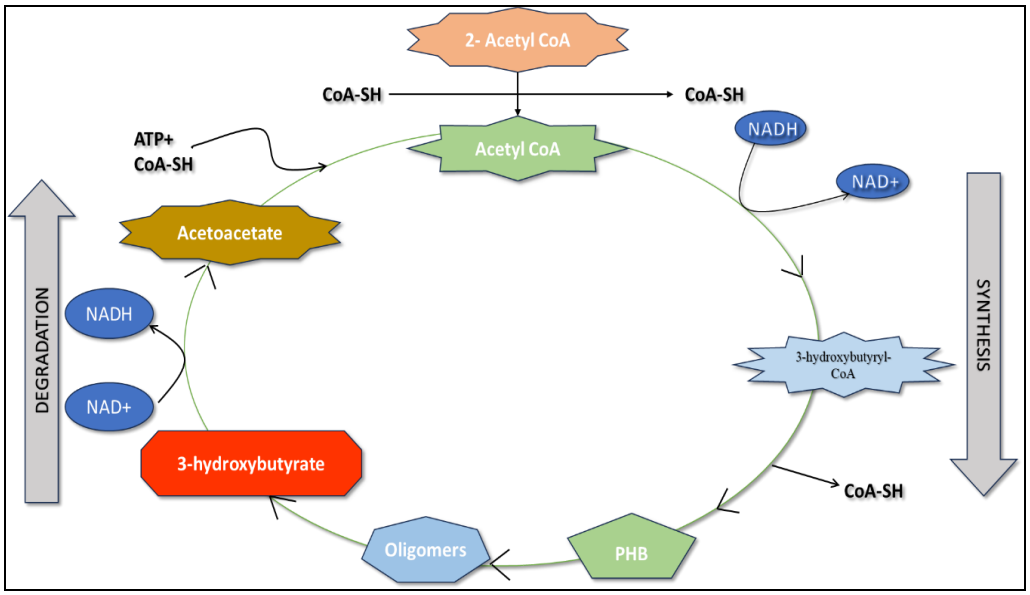

Fig 2: Polyhydroxybutyrate (PHB) synthesis and degradation process (33)

PHA production from Microorganisms

Numerous bacterial species have been identified as capable of accumulating polyhydroxyalkanoates (PHA) (13). PHA has been reported from diverse habitats, including soil, sewage sludge, marine sediments, ponds, mangrove ecosystems, and gas field soil [14, 15].

PHB production by bacteria

The isolation and characterization of bacteria capable of producing PHA, and the identification of highly proficient PHA producers, have been conducted on *Sphingomonas sp.* as confirmed by 16s rDNA sequencing. In their investigation, the strain was cultivated using various sugars and organic acids to assess its capacity for PHA accumulation. The strain demonstrated the ability to accumulate PHA when cultured with disaccharides, aldohexoses, sugar alcohols, and certain organic acids. However, it exhibited an inability to utilize ketoses, pentoses, and starch. Notably, among the sugars examined, sucrose or mannose yielded a high PHA production, constituting approximately 55-60% of the cell biomass [16]. Carbon sources such as acetate and butyrate were identified as optimal for the accumulation of polyhydroxybutyrate (PHB). Interestingly, the presence of nitrogen sources in the growth medium was observed to impede PHB accumulation despite promoting growth. Furthermore, conditions of limited phosphate and sulfate were found to enhance polymer accumulation by the isolates [17].

Table 1: Accumulation of PHB (%) and dry cell weight (DCW) obtained in different

Microorganisms	Source of C/N	DCW (g/l)	PHB (%)	References
Bacillus mycoides RLJ B-17	Saccharose	3.6	69.4	38
	Glucose	3.2	56.6	
	Fructose	2	55	
Bacillus megaterium	Glucose	7.1	59.1	39
	Glycerol	7.7	62.4	
Bacillus megaterium BA-019	Saccharose/Ammonium sulfate	1.96	28.57	40
	Saccharose/Urea	2.83	30.2	
	Molasses/Ammonium sulfate	6.2	49.92	
	Molasses/Urea	7.05	55.46	
Cupriavidus necator	Commercial glycerol	82.5	62	41
	Glycerol residue	68.8	50	42
Cupriavidus necator DSM 545	Corn starch with soybean oil	11.64	43	43
Cupriavidus necator DSM 545	Glucose + glycerol	68.5	64.5	44
Cupriavidus necator H16	$NH_4H_2PO_4$	3.3	87.9	
	Urea	8.2	75.6	
Rasltonia eutropha ATCC 17697	Fructose	7.5	66.2	45
Zobellella denitrificans MW1	Glycerol	88.2	66.9	46
Alcaligenes latus	Maple sap	4.2	77	47
Azotobacter chroococcum 23	Starch	8.4	44	48
Halomonas TD01	Glucose	40	60	49
Methylobacterium organophilum NCIB 11278	Methanol	130	56	50

Microorganisms under different conditions

PHB production by cyanobacteria

Under varying cultivation parameters, microorganisms such as cyanobacteria can double their biomass within 24 hours [18]. PHB, a biopolymer, is synthesized by cyanobacteria in response to an augmentation in carbon availability alongside restricted nitrogen or phosphorus concentrations [9, 19]. Cyanobacteria possess the ability to utilize light energy, carbon dioxide (CO_2), and water (H_2O) to biosynthesize organic compounds via the process of photosynthesis [20, 21]. When carbon dioxide (CO_2) is employed as a nutrient source for extensive cultivation of cyanobacteria aimed at producing PHB on a large scale, this process aids in mitigating the environmental impact associated with CO_2 emissions into the atmosphere [22, 23, 24]. The comparatively low weight of polyhydroxyalkanoates (PHA) within cyanobacteria, in contrast to other bacterial counterparts, likely arises from their small size and mass. Additionally, it has been suggested that the capacity for PHA synthesis in cyanobacteria closely resembles that observed in the majority of bacterial species found in natural environments [25].

Table 2: The sequential steps involved in polyhydroxybutyrate (PHB) biosynthesis (26)

Step	Enzyme	Substrate	Product
Condensation	β-ketothiolase (phaA)	2 Acetyl-CoA	Acetoacetyl-CoA
Reduction	Acetoacetyl-CoA reductase (phaB)	Acetoacetyl-CoA	3-Hydroxybutyryl-CoA (3HB-CoA)
Polymerization	Acetoacetyl-CoA reductase (phaB)	3-Hydroxybutyryl-CoA (3HB-CoA)	Polyhydroxybutyrate (PHB)

PHB production by yeasts

To learn more about PHB synthesis in eukaryotes, yeast cells were employed as models for the production of PHBs; however, developing new pathways appears to be a useful stiff crystalline substitute. Yeasts offer distinct advantages over bacteria as hosts for PHB synthesis. Certain yeast species such as *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, and *Candida utilis*, among others, have been recognized as generally recognized as Safe (GRAS) microorganisms by the Food and Drug Administration (FDA) [25].

In the production of PHB homopolymers, two transgenic yeast strains have been developed. In one strain (*Saccharomyces cerevisiae* INVSc1/PHA1), the genes encoding PHB synthase from *Ralstonia eutropha* are introduced into the cytoplasm. Conversely, in the second strain (*Schizosaccharomyces pombe* Q01/PHB), the genes responsible for PHB biosynthesis are integrated into the chromosome [25].

PHA production by recombinant bacteria

PHAs are commonly classified into two primary groups: short-chain-length PHAs (PHASCL) and medium-chain-length PHAs (PHAMCL). PHASCL consists of repeat units composed of hydroxy fatty acids containing three to five carbon atoms, while PHAMCL contains repeat units comprising hydroxy fatty acids with six or more carbon atoms. Due to their biodegradable and biocompatible characteristics, PHAs have been extensively investigated as promising alternatives to petroleum-derived polymers in various fields including medicine, drug delivery, agriculture, horticulture, the fibers industry, and consumer products [27].

Escherichia coli, being the most well-known bacterium, serves as an ideal host for PHA production. It is amenable to heterologous expression of foreign genes, allowing for facile manipulation and enhancement through recombinant DNA techniques or metabolic engineering. Moreover, high-cell-density cultivation methods for numerous *E. coli* strains are well-established. Metabolic pathways for both PHASCL and PHAMCL in *E. coli* have been established for over a decade. *E. coli* cells accumulating substantial quantities of PHASCL tend to become fragile, simplifying the isolation and purification of the biopolymer. Additionally, this bacterium does not produce PHA-degrading enzymes [4]. The utilization of recombinant *E. coli* for PHA production has been limited both in laboratory and industrial settings due to inefficiency and high expenses. Specifically, in the production of polyhydroxybutyric acid (PHB), approximately 40% of the overall production costs are attributed to raw materials [28]. Therefore, there is a necessity for employing more economical carbon sources to alleviate the substantial production expenses associated with PHAs. These polymers have demonstrated efficacy across diverse wild strains, necessitating a cost-effective substrate for their synthesis [29].

Recombinant strains of *E. coli* carrying the PHA biosynthesis genes from *Alcaligenes eutrophus* within a stable, high-copy-number plasmid have been engineered and utilized for achieving high productivity in PHA production. Given *E. coli*'s ability to metabolize diverse carbon sources like glucose, sucrose, lactose, and xylose, further cost reductions in PHA production are feasible through the utilization of more economical substrates such as molasses, whey, and hemicellulose hydrolysate. While natural PHA-producing bacteria typically exhibit prolonged generation times and have relatively low optimal growth temperatures, rendering them challenging to lyse and often possessing pathways for PHA degradation, bacteria like *E. coli* lack inherent PHA synthesis or degradation capabilities. Nonetheless, *E. coli*'s rapid growth rate, even at elevated temperatures, and susceptibility to lysis facilitate the accumulation of significant polymer quantities. The ease of cell lysis also contributes to cost savings in PHA granule purification processes [30]. *E. coli* has been employed for the transference of PHA genes, with PHB production primarily investigated in recombinant *E. coli* cells containing PHA synthesis genes sourced from *Ralstonia eutropha* [31].

PHB Fermentation Methods

Various fermentation methodologies can be employed to produce polyhydroxybutyrate (PHB). These encompass discontinuous techniques like batch culture, fed-batch culture, and repeated fed-batch culture [34]. Continuous fermentation approaches such as continuous fed-batch systems utilizing gaseous substrates, single-stage chemostat process, two-stage chemostat process, and multi-stage chemostat process within continuously stirred tank reactor (CSTR)-bioreactor cascades are also viable methods for PHB production [35]. It is crucial to highlight that there are fundamental distinctions in the feasibility of continuous processes for generating extracellular products, as outlined earlier, versus intracellular products like PHB, which are the

focus of this review [36]. The batch culture method is a simple yet inefficient process used in PHB material production. In this method, fermentation occurs in discrete batches, with limited availability of nitrogen and carbon sources during the initial stages due to physiological constraints of the production strain [35].

Challenges and Outlooks

The utilization of bio-plastics sourced from bacterial sources represents a promising avenue for sustainable and economically viable polymer production. This methodology hinges on leveraging microorganisms to biosynthesize polymers using cost-effective substrates derived from agricultural and food waste. However, this approach presents inherent challenges. Firstly, there is a necessity to identify the most suitable microbial strain capable of thriving under optimal conditions while demonstrating high productivity and specificity towards the desired product. Often, genetic manipulation is required to enhance yield or tailor a microorganism's substrate consumption [24]. Secondly, fermentation processes demand precise control over environmental parameters such as temperature, pH, substrate concentration, and oxygen availability, which poses significant challenges in scaling up production due to the associated costs. Ensuring that microbial cultures can effectively metabolize selected substrates to produce polymers at desirable rates necessitates meeting optimal conditions for bacterial growth and polymer biosynthesis [37].

Conflict of Interest

Not available

Financial Support

Not available

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