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Poly-β-hydroxybutyrate: A Biodegradable Polyester, Biosynthesis and Biodegradation

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Authors' contributions

This review article was written in collaboration between both authors. Author MS designed the study, managed literature searches and wrote the first draft of the manuscript. Author HKD managed the analyses of the review and literature searches. Both authers read and approved the final manuscript.

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ABSTRACT

Bioplastic can be degraded in nature with time and produce carbon dioxide as an end product. It reduces the environmental pollution created by synthetic plastic. Biodegradable plastic can be used to make plastic bags and also have application in medical field like in formation of bone implants. Poly-β-hydroxybutyrate is a microbial polymer formed inside the bacterial cell and commercially important in formation of biodegradable plastic. In this review we tried to summarized identification, biosynthesis, metabolism, biodegradability and applications of PHB. Information summarized in review can lead to design many experiments to increase production of bacterial polymer as valuable renewable products.

Keywords: Synthetic plastic; biodegradable plastic; poly-β-hydroxybutyrate; inclusion body.

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1. INTRODUCTION

The history of plastic begins from 1862 when Alexander Parkes introduced these. According to the statistics in 2000, the annual consumption of plastics in India was approximately 4 kg / person / year, and that of world average of 24.5 kg/ person/year [1].

Plastic plays important role in our day to day life. It is used to prepare simple plastic carry bags to complex surgical and medical implants. Petroleum based synthetic plastics are mainly used for manufacturing of many valuable products. Those are used in daily routine life. Nowadays, modern life style contributes in high consumption of plastic, everything is available in packed form like bottle packed water, milk, juices, coke, instant vegetables as of which requirement of synthetic plastic (polyethylene) increases. The high requirement of synthetic plastic increases the garbage that is non biodegradable. The management of these synthetic plastics increases the burden on the municipal department of the country. Municipal department discards plastic by dumping and incineration, which will make these dumping lands barren or cause the gaseous pollution in the environment. Synthetic plastic has adverse effect on the environment and on the health of grazing animals. Accumulation of non-degradable plastic bags in the environment is one of the major causes of pollution now-a-days. Only 1-2% of plastic bags end up getting recycled. Thousands of marine animals and more than one million birds die each year as a result of plastic bags [2]. Often, pieces of plastic litter ingested by animals, clogging their intestines which results in the death by starvation. Birds become entangled in plastic bags and can't fly as a result finally die.

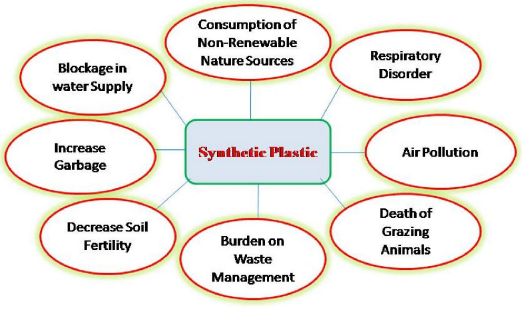

Fig. 1. Adverse effect of petroleum based plastic on environment, human health and grazing animals

Therefore, it is not justified the use of long lasting plastic for short lived applications. In response to the hazardous effects of petroleum based plastics, environmental friendly biodegradable plastic has been introduced. Polyhydroxy-alkanotes (PHA) is a type of polymer that has been used as biodegradable plastic [3]. Polyhydroxybutyrate (PHB) is most widely used PHA that was discovered by French Scientist Maurice Lemoigne in 1925 from bacteria *Bacillus megaterium* [4]. Commercial processes for PHA production were initially developed by W. R. Grace in the 1960s and later developed by Imperial Chemical Industries, Ltd., U.K. in the 1970s and 1980s [5]. In 1996, Monsanto sold PHB by the name of Biopol. It has similar physical properties with polypropylene. Due to this character, PHB has attracted the vision of researcher towards its study and production. Another reason for gaining priority is that the use of these biodegradable and biobased plastics will definitely reduce the pollution caused by carbon dioxide emission from plastic wastes [6]. Various scientists are working on different area related to PHB like increased production of PHB, genes involved in PHB synthesis and its applications. Here in this review, work done on these aspects related to PHB like identification, biosynthesis, metabolism, biodegradability and applications has been summarized.

2. POLY-β-HYDROXYALKANOTE (PHA)

PHAs are a family of optically active biopolymers that are fully biodegradable. PHAs are biodegradable, biocompatible and thermoplastic polyesters produced by various microorganisms. The most extensively studied PHAs are the PHB and can be produced in high yield by fermentation of a variety of bacterial strains. Its copolymers with varying ratios of hydroxyvalerate (HV) are the most widely used members. The copolymers of hydroxybutyrate (HB) with hydroxyvaleric (HV) acid are less crystalline, more flexible and more readily processable PHB itself. In all, PHAs have been characterized so far, the hydroxyl- substituted carbon atom is of the R configuration, except in some special cases where there is no chirality. However, this alkyl side chain is not necessarily saturated: aromatic, unsaturated, halogenated, epoxidized and branched monomers have been reported as well.

The variation in the alkyl substituent, the position of the hydroxyl group is somewhat variable

and 4-, 5- and 6-hydroxy acids have been incorporated [7]. Many factors affect the PHAs chemical composition like the microbial strain, the substrate, the cultivation condition, the extraction method, the number of *phaC*, *phaB* genes, the regulator phasin and the presence of inhibitors. They inhibit different pathways, especially those which supply the synthases with different kinds of monomer or inhibit other pathways, which consume these monomers for their own or degrade it to shorter units like β- oxidation pathway [8]. PHB has been studied most extensively and created the commercial interest in these classes of polymers. PHB is the most common type of PHA and the ability of bacteria to accumulate PHB is often used as a taxonomic characteristic [9].

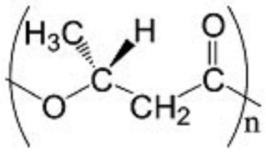

Fig. 2. Structural formula of PHA

Table 1. Types of PHAs

N	R	Type of monomer
1	Hydrogen	Poly(3-hydroxypropionate)
1	Methyl	Poly(3-hydroxybutyrate)
1	Ethyl	Poly(3-hydroxyvalerate)
1	Propyl	Poly(3-hydroxyhexanoate)
1	Pentyl	Poly(3-hydroxyoctanoate)
1	Nonyl	Poly(3-hydroxydodecanoate)
2	Hydrogen	Poly(4-hydroxybutyrate)
2	Methyl	Poly(4-hydroxyvalerate)
3	Hydrogen	Poly(5-hydroxyvalerate)
3	Methyl	Poly(5-hydroxyhexanoate)
4	Hexyl	Poly(6-hydroxydodecanoate)

3. POLY-β-HYDROXYBUTYRATE AS INCLUSION BODY

Polyhydroxybutyrate found in approximately 75 different Gram negative and positive bacterial genera as a carbon and energy storage granules. Polyhydroxybutyrates (PHB) are synthesized and accumulated as storage granules in various bacteria like *Rhizobium* sp., *Bacillus megaterium*, *Azotobacter* sp. and *Pseudomonas* sp. [10,11,12].

PHB granules accumulation starts in response to adverse conditions like a nutrient deficiency (lack of N, S, P) and these granules can be utilized as a source of nutrients which prevent the bacterial cell death during starvation conditions [13,14]. In the absence of nitrogen, PHB synthesis generally increases. During nitrogen starved conditions, reduced amino acid synthesis accompanied by the increased Acetyl CoA and increased activity of β-ketothiolase, which promote PHB accumulation. However the intermediate process and regulation mechanisms are yet to find out. In this starved condition, a concentration of acetyl phosphate increases and finally PHB synthase enzyme is activated [15]. The size of PHB granules in *R. eutropha* and other PHB accumulating bacteria are in the range of 0.2 to 0.5μm and the cell size is a little smaller than the average cell diameter. PHB granules are composed of spherical shell-core particles of a PHBs core enclosed by phospholipids and proteins [8]. A self assembly process initiates and leads to the formation of insoluble cytoplasmic inclusions with a phospholipid monolayer and covalently attached PHBs synthases at the surface. The structural protein called “phasing” can be found attached to the granule surface. Generally, PHB granules are present in the center of bacterial cell but it was found PHB granule formation can be possible in the cell periphery.

Table 2. List of PHB producing bacteria and different isolation sites

Sr. no.	Isolation sites	Isolates	References
1.	Sewage treatment plant	*Bacillus megaterium*	[49]
2.	Garden soil	*Bacillus thuringiensis* IAM 12077	[50]
3.	Tannery effluent	*Rhodobacter capsulatus*	[14]
4.	Sugar industry effluents	*Saccharococcus thermophilus*	[47]
5.	Sewage sludge soil	*Staphylococcus spp*	[51]
6.	Industrial and agricultural land	*Bacillus subtilis* *Pseudomonas sp*	[52,53]

Some non storage PHB granules with low molecular weight present in a cell membrane that play important role in voltage gated or calcium gated ion channels and protect the macromolecules from its degradative enzymes [16].

4. PHYSICAL AND CHEMICAL PROPERTIES OF PHB

Like any other polymer, chains of monomers in PHB form either homopolymer or heteropolymer. Generally PHB known to be linear has composed of 3-hydroxy butanoic acid monomer units, each unit from an ester bond with the hydroxyl group of the other one.

PHB has an extremely regular structure, all the side groups on the polymer chain point in the same direction. This causes the chains to form helical structures, with the side groups all pointing away from the centre of the helix to minimize steric hindrance. Molecular weight of PHB from bacteria is usually in the range 1×10^4 – 3×10^6 $g\cdot mol^{-1}$ [17]. PHB is very low strength biomolecule that need to improve. High molecular weight PHBs are useful for producing films and strong fibers. The molecular weight of PHBs depends upon the PHB synthases, which vary in their substrate specificity short chain length (scl) monomers (C3-C5) and medium chain length (mcl) monomers (C6-C14) [18]. Carbon sources play a key role in diversification of PHB production. These carbon sources include saccharides, n-alkanes, n-alkanoic acids and n- alcohols. Wastestreams like frying oil, vinegar, fats, food, agriculture, waste water and oil mill effluents which provide a free source of carbon for PHB production [19]. The PHB composition also depends on the metabolic pathway involved. The molecular weights have been measured by light scattering, gel permeation chromatography and sedimentation analysis. Their monomer composition has been determined by gas chromatography, mass spectroscopy and nuclear magnetic resonance analysis. PHBs crystal structure, polydispersities, melting point, enthalpy of fusion, glass transition temperature and mechanical properties were established by the use of different procedure such as differential scanning chromatography. PHB, compared with the plastic like polypropylene, is considered as having the most common properties. Like, PHB is insoluble in water and relatively resistant to hydrolytic degradation, but soluble in organic solvents such as chloroform and other chlorinated hydrocarbons. In the case of PHB-within the cell it exists in a fluid, amorphous state. However, after extraction from the cell, PHB becomes crystalline and rigid but brittle material. The brittleness of PHB during mechanical processes makes it unresisting to stress. There is another monomer included in the structure of PHB such as 3HV (3-hydroxyvalerate) which decreases PHB crystallinity and increases its elasticity [20]. The glass transition temperature and melting temperature of PHB is around 4°C and 180°C respectively. The major problem of PHB is that it decomposes near its melting point. The density of amorphous PHB is 1.18 gCm^{-3} and for crystalline PHB density is 1.26 gCm^{-3}. Mechanical properties like the Young's modulus (3.5 GPa) and the tensile strength (43 MPa) of PHB are close to those of polypropylene. The extension to break (5%) of PHB is; however, lower than extension to break of polypropylene (400%).

Table 3. A comparison of physical of polypropylene and PHB

Physical property	Polypropylene	PHB
Melting point (ºC)	171-186	171-182
Glass transition point (ºC)	5-15	5-10
Crystallinity (%)	65-70	65-80
Density (g/cm^3)	0.905-0.94	1.23-1.25
Molecular weight (10^5)	2.2-7	1-8
Flexural modulus (GPa)	1.7	3.5-4
Tensile strength (MPa)	39	40
Extension to break (%)	400	6-8
Ultraviolet resistance	Poor	Good
Solvent resistance	Good	Poor
Oxygen permeability (cm^3/m^2.atm.d)	1700	45
Biodegradability	None	Good

Overall PHB is stiffer and more brittle plastic material compared to polypropylene. The brittleness is due to the formation of large crystalline domains in the form of spherulites [21]. Ultra-high molecular PHB was prepared by transgenic *Escherichia coli* with genes from *Cupriavidus necator*. It has high mechanical properties then regular PHB [22].

5. IDENTIFICATION OF PHB

Sudan black, carbon fuschin, Nile blue and Nile red can be easily used for PHBs detection. Sudan black is most common stain used for screening of PHA accumulating bacteria. Sudan black staining is very simple and reliable process for identifying the presence of PHB granules in bacterial cell [8]. Subsequently, Burdon et al. [23] confirmed the greater value of this dye and modified the procedure for demonstrating intracellular fatty material in bacteria by preparing microscopic slides of bacteria stained with alcoholic Sudan black B solution and counterstained with safranin. Lewis [24] gave the procedure for demonstrating intracellular fatty material in bacteria by preparing, from suspensions of the organisms in alcoholic Sudan black B solution, dried films counterstained with safranin. Previously, it was thought that dried, fixed films were entirely unsuitable for fat stains.

Besides Sudan black B dye Nile blue A dye also be used for identification of PHB. Ostle and Holt [25] reported that Poly-β-hydroxybutyrate granules exhibited a strong orange fluorescence when stained with Nile blue A. For treatment of heat-fixed cells with 1% Nile blue A the excitation was observed at an excitation wavelength of 460 nm. Glycogen and polyphosphate did not stain. Nile blue A stain appeared to be a more specific stain for poly-β-hydroxybutyrate than Sudan black B.

On medium plate, PHB producer colonies can be differentiated from those of non producers. Kranz et al. [26] described the colony screening and selection systems to analyze the production of PHAs in *R. capsulatus*. Screening with Nile red dissolved in acetone distinguished between PHA producers and non producers. Spiekermann et al. [27] recommended the use of a sensitive viable colony staining method using Nile red for the direct screening of bacteria that accumulate PHA. The direct inclusion of 0.51 g/ml of dye in the medium did not affect the growth of the cells but allowed the detection of the presence of PHAs in the viable colonies at any time during growth. The stained PHA producer colonies exhibited strong fluorescence when observed under UV light.

6. BIOSYNTHESIC PATHWAY OF PHB

PHB is produced by the cell in response to a nutrient limitation in order to prevent starvation. Deficiency of nutrients like magnesium, sulfate, phosphate and nitrogen initiates the biosynthesis of PHB [28]. Besides it, some bacteria like *Alcaligenes latus* and *Azotobacter vinelandii* accumulate PHB even in the absence of nutrient limitation [29,30]. PHB is a carbon storage polymer and its synthesis is also induced by high carbon to nitrogen ratio.

PHB production is initiated by diversion of acetyl CoA from the TCA cycle due to the suppression of citrate synthase and iso-citrate dehydrogenase (key enzymes of TCA cycle) activities leading to blockage of TCA cycle. High acetyl CoA concentration and low CoA activates β-ketothiolase which converts acetyl CoA to aceto-acetyl CoA, which is then converted to 3-hydroxybutyryl CoA through the action of aceto-acetyl CoA reductase [31].

Intracellular concentration of acetyl-CoA play important role in polymer synthesis. The nutrient limitation stimulates a metabolic pathway, which shunts acetyl units from Krab's cycle into the production of PHB. NADH is generated and used for cell growth. When growth ceases, NADH concentration increases, which reduces the activity of the TCA cycle enzymes citrate synthase and isocitrate dehydrogenase. TCA cycle is inhibited and acetyl-CoA enters PHB synthetic pathway [32]. Under the nutrient rich conditions, the production of high amount of coenzyme A from TCA cycle blocks PHA synthesis by inhibiting β-ketothiolase. The acetyl CoA is channeled into the TCA cycle for energy production and cell growth. Biosynthesis pathway of PHB formation was first discovered by Stanier and coworkers in 1959 [4]. Genes coding for proteins involved in the biosynthesis of the PHA referred in alphabetical order as phaA, phaB, phaC. Mainly three enzymes play important role in the PHB synthesis viz ketothiolase, reductase and synthase encoded by *phaA*, *phaB* and *phaC* respectively.

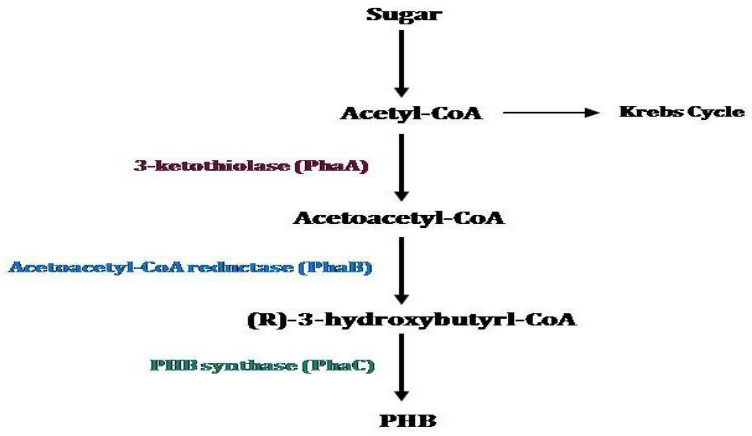

Fig. 3. Genes involved in PHB synthesis

Table 4. Genes involved in PHB biosynthesis

S. no.	Gene	Protein	References
1.	*PhbA*	β keto thiolase	[47]
2.	*PhbB*	Acetyl CoA reductase	[47]
3.	*PhbC*	PHB synthase	[47]
4.	*PhbP*	Phasin	[48]
5.	*PhbZ*	PHB depolymerase	[48]

The first two enzymes identified by Schlegel and Dawes in 1973 [33,34] and the third enzyme synthase was identified and characterized by Merrick and co-workers in 1964 [35]. PHB synthesis pathway is a very complex process and its regulation involves different environmental, metabolic and genetic signals. An excess of acetyl CoA reduces the synthesis of PHB. In *B. megaterium* synthase present as inactive molecule which became activated by different polypeptides that produced by the gene *phaR* [13] *phaZ* and *phzP* also participate either in the catabolism (*phaZ*) or in the stabilization (*phaP*) of the PHB granules. The protein which is called as phasin is a low molecular weight protein that regulates the size, number and surface to volume ratio of PHB by binding to inclusion granules. These properties are also regulated by amount of *phaC* present in cells [33].

7. METABOLISM OF PHB

The pathway for PHB synthesis begins with the condensation of two molecules of acetyl-CoA by β-ketothiolase, which is encoded by the *phaA* gene. This product is reduced to D-(-)-3-hydoxybutyryl –CoA by acetoacetyl CoA reductase product of *phaB*. PHB synthase catalyses the polymerasation of PHB monomers through the use of two thiol groups. The activity of PHB synthase has been studied in detail and residues found to be highly conserved across PHB-producing microorganisms. These sites are: Ser-260, Cys-319, Gly-322, Asp-351, Trp-425, Asp-480, Gly-507, and His-508. Cys-319 and Gly-322 are part of the G-x-C-x-G-G motif that is required for the catalytic activity of the enzyme. When the highly conserved Cys-319 residue is mutated, PHB synthase activity is lost, suggesting that it is one of the thiolate groups [34]. The enzyme forms a dimer with the first thiol group (Cys-319) on one subunit acting as the loading site, and the same thiol group on the other subunit serving as the elongation site. The first thiol group covalently binds to D-(-)-3-hydroxybutyryl-CoA, resulting in the release of Coenzyme A. Similarly, the corresponding thiol group on the other subunit covalently binds to another molecule of D-(-)-3-hydroxybutyryl-CoA and cleaves the coenzyme A on that molecule. The subsequent D-(-)-3-hydroxybutyryl attached to the second thiol group is then subjected to nucleophilic attack. This activates the D-(-)-3-hydroxybutyryl and results in a transesterification reaction that attaches the D-(-)-3-hydroxybutyryl on the first thiol group to the end of the monomer bound to the second thiol group. The elongation

process occurs several thousands of times to create polyesters of high molecular weights [36].

8. DEGRADATION OF PHB

The degradation time depends on the composition, as well as the environment. It can be as little as a few months, for example, in a sewage works, where there are a lot of bacteria to feast on the polymer. In the sea, where there are fewer bacteria, it takes a bit longer, may be a few years, but sitting on a shelf, for example at home in the kitchen or bathroom, the plastic will last for decades. Biodegradable plastics undergo degradation by the enzymatic action of microorganisms such as bacteria, fungi and algae. PHB sinks in water and sinking facilities its anaerobic biodegradation in sediments. The names of genes involved in PHBs degradation are referred to opposite alphabetical order such as *phaZ*, *phaY*, *phaX*, *phaW* etc [37]. Degradation of PHB is started intracellularly by the action of PHB depolymerase. The first structural gene of a PHA depolymerase (*phaZ*) was cloned and sequenced in 1989 by Saito et al. [38]. PHA depolymerase is carboxyesterases and hydrolyze the water insoluble polymer to water soluble polymer and finally to water and carbon dioxide. PHB degrading bacteria were isolated on the basis of utilization of PHB as a sole source of carbon and energy. PHBs degrading bacteria differ from each other depending on the type of PHAs they degrade. However, some bacteria revealed a rather broad polyester specificity and are able to utilize a wide range of PHAs. Fungi also could degrade PHAs and their depolymerases have been characterized. Fungal depolymerases show a similar characteristic with bacteria, they are stable at range of pH, temperature, inhibited by reducing agents such as di-thiothreitol, which indicates the presence of essential disulfide bonds. Depolymerases have three strictly conserved amino acids: serine, aspartate and histidine. The serine is part of the lipase-box pentapeptide Gly-Xaa1-Ser-Xaa2-Gly. The oxygen atom of the serine side chain is the nucleophile that attacks the ester bond. The activities of PHAs depolymerase may vary depending on the composition and the physical form of the polymer, the environmental conditions and the dimension of the sample [39].

The gene *phaZ* which release the D-3-hydroxybutyrate monomer is oxidized by the action of 3-hydroxybutyrate dehydrogenase, to acetoacetate which is esterified to acetoacetyl CoA synthetase. Acetoacetyle CoA is hydrolyzed into acetyl-CoA by *phaA* that is assimilated via Krab's cycle. Alternatively, upon cell death and lysis, PHB granules are released into the extracellular environment where they undergo a transition into a partially crystalline polymer and can be broken down by the action of extracellular PHB depolymerases [40].

9. APPLICATION OF PHB

PHB has many different applications like they were used for the fabrication of bottles, fibers, latex and several products of agricultural, commercial or packaging interest [13].

In industries PHB alone or with 3HV as a copolymer have been used as a diaper back sheet and films. PHBs have also used for non-woven fabric material. It has been used as hot melt adhesives and as toner in ion-conducting polymer. PHBs are used as latex in paper coating applications, in dairy cream substitutes, in food flavor delivery agents and as raw materials for the synthesis of chemicals and paints. PHB latex is sprayed onto a substrate to produce coating [41].

In agriculture PHB can be used for release of pesticides. PHB in nitrogen fixing bacteroids plays an important role in nitrogen fixation efficiency that may be due to the participation of *phaP* gene in nodule formation [42].

Unlike the Gram negative bacteria, the polymer produced by *Bacillus* sp. are free from endotoxins and can be used for biomedical applications. These polyesters are used for medical applications such as sutures, implants, urological stents, neural and cardiovascular-tissue engineering, fracture fixation, treatment of narcolepsy and alcohol addiction, drug delivery vectors, cell microencapsulation, support of hypophyseal cells, or as precursors of molecules with anti-rheumatic, analgesic, radiopotentiator, chemopreventive, antihelmintic or anti-tumuoral properties. For all medical applications biocompatibity is a basic required that has been fulfilled by PHB [43]. To check the biocompatibility of PHB, the structural organization of cellular molecules involved in adhesion was studied using osteoblastic and epithelial cell lines. On PHB, both cell lines revealed a rounded cell shape due to reduced spreading. PHB-co-PHV film was chosen as a temporary substrate for growing retinal pigment epithelium cells as an organized monolayer

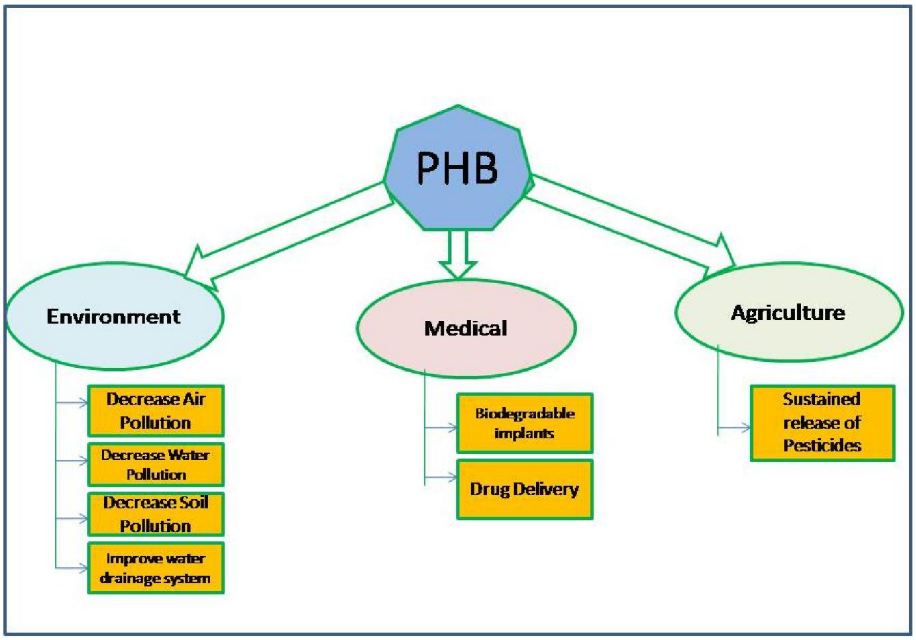

Fig. 4. Application of PHB

before there subretinal transplantation. The polymer film was rendered hydrophilic by oxygen plasma treatment to increase the reattachment of D407 cells on the film surface. PHB nonwoven patches were implanted as transannular patches into the right ventricular outflow tract and pulmonary artery in 13 weanling sheep. It was concluded that PHB non-woven patches can be used as a scaffold for tissue regeneration. The regenerated vessel had structural and biochemical qualities in common with the native pulmonary artery. PHBs have been used in tissue engineering, as antibiotic carrier and many other mechanical applications. PHBs possess the biodegradability, biocompatibility and thermal properties which show a promising future in pharmaceutical application such as drug delivery, which open a new approach [44,45]. Chemical modifications such as chlorination, cross-linking, epoxidation, hydroxylation, and carboxylation have been made to yield further functionalized PHBs [46].

Although biodegradable plastics might seem an environmentally friendly alternative to non-biodegradable plastics, there are some disadvantages. Degradation of oil-based biodegradable plastic may result in the release of carbon dioxide, a harmful greenhouse gas that participates in global warming. Degradation of starch-based biodegradable plastics may not have harmful byproducts such as CO_2 gas. However, they have the potential to contaminate soil and water [34].

10. CONCLUSION

The demand for biodegradable plastics continues to grow in future. It is affected by several factors including: the continued increase of crude oil prices, consumer demand for more environmentally friendly products, and increased restrictions on the use of nondegradable plastic products. To avoid disrupting the ecosystem, plastics needed to be recycled, without creating chemical or biological imbalances. Another benefit of producing plastics from renewable resources is the reduction of fossil fuel derived CO_2.

The production of monomers and polymers by enzymes, microbes, and plants represents a cleaner and more sustainable process. The progress of techniques, especially genetic engineering, will allow microbes and plants to produce biodegradable polymers or their monomers more efficiently from inexpensive biobased and renewable carbon sources. Recent research highlights the molecular and biochemical process involved in formation of biopolymer inclusion inside the microbial cell. Intracellular inclusions are increasingly identified as valuable nanostructures for application in various fields such as medicine, agriculture and industry.

In this review, efforts have been made to highlight a better understanding of polymer biosynthesis and substrate selection that can lead to increased production of bacterial polymer as valuable renewable products.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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