

# Microbial biopolymers: A initiative step towards green plastic

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## Abstract

At every stage of existence of synthetic plastic i.e. manufacturing, use and disposal it creates toxic pollution. Recent issues concerning the global environment pollution, human health and solid waste management have created lots of concern for the development of biodegradable plastics. In this regards Poly- $\beta$ -hydroxy butyrate (PHB) has been found as green and biodegradable thermoplastic having similar properties to various synthetic polymers. Moreover, under aerobic and anaerobic conditions, it is fully degraded to water and carbon dioxide. This review article focuses on the screening, production and degradation of biopolymer for its possible use as biodegradable and eco-friendly polymer to hazardous synthetic polymers.

**Keywords** – Biodegradation, Bioplastic, PHB, Poly- $\beta$ -hydroxybutyrate

## 1 INTRODUCTION

Day by day drastic increase in the use of synthetic thermoplastics due to establishment in science and technology. Shimao (2001) [1] reported that each year millions and trillions of synthetic plastics are synthesized and discarded in the environment. This synthetic polymer are resistant to chemical, physical and biological degradation process and hence causes discarding issues after their use. The presence of synthetic plastic in the surroundings causes soil, water and air pollution. It also blocks different drainage system as well as wastewater treatment plants. Moreover it disturbs the food chain in the environment. Disposal of synthetic polymers and agricultural plastic wastes have been considered as major solid waste in the environment. For discarding of municipal solid waste, landfilling is the most suitable method in which garbage is buried in the form of layers under the soil. For the disposal of agricultural plastic wastes, microbial degradation is best alternative method. The plastic industry worldwide has invested \$1billion to support increased recycling, and to educate communities [2]. In order to prevent the solid waste problems created due to synthetic polymers in the environment, the development of biodegradable plastic for consumer products is of great concern [3], [4].

## 2 SYNTHETIC PLASTICS

Synthetic plastic composed of a variety of additives including toxic compounds such as adipates and phthalates. These additives make the plastic more reliable for packaging in food industries as it

### 3.2 Polylactic acid (PLA)

brittle the plastic material. Also in making toys, cups, bottles, utensils and many other items synthetic polymers are used. Smaller particles of additives spread out from the product and proven to be toxic. Teuten et al. (2009) [5] reported that the use of di-2-ethylhexyl phthalate and other phthalates such as alkylphenols are banned because of their toxicity in some applications. Some food packaging materials are investigated such as polystyrene and it is stated that it is cause of hormonal malfunctioning in human beings and are suspected as carcinogens [6].

## 3 BIOPOLYMER

Bio-plastic is a form of plastic synthesized from renewable resources such as plant starch and microbial species. Bioplastics are made from a compound called polyhydroxyalkanoate (PHA).

### 3.1 Poly-hydroxyalkanoate (PHA)

PHAs are linear polyesters produced in nature by bacterial fermentation of sugar or lipids. They are produced by the bacteria to store carbon and energy. Polyesters are deposited in the form of highly refractive granules in the cells. Depending upon the microorganism and the cultivation conditions, homo or copolyesters with different hydroxyalkanoic acids are generated. The family of PHAs includes several polymeric esters such polyhydroxybutyrates (PHB), polyhydroxybutyrate cohydroxyvalerates (PHBV), polyhydroxybutyrate cohydroxyhexanoate (PHBHx) and polyhydroxybutyrate co-hydroxyoctanoate (PHBO). PLA is biodegradable thermoplastic aliphatic polyester derived from renewable resources such as corn starch,

sugarcane etc. PLA is prepared by Ring Opening Polymerization (ROP) of lactide, a cyclic dimer of dehydrated lactic acid that is produced by fermentation [7], [8]. Another way to prepared PLA is the direct condensation of lactic acid monomers. Its characteristics are similar to conventional petrochemical based plastic. It is used in plastic processing industry for the production of films, fibers, plastic containers, cups and bottles. PLA has the capability of producing hybrid paper-plastic packaging that is compostable and also has the ability to recycle back to lactic acid by hydrolysis. In other hand Polybutylene Succinate (PBS) and Polytrimethylene Terephthalate (PTT) are also synthesized using chemical reactions.

Soam et al. (2012) [9] stated that among all these PHA, Poly  $\beta$ -hydroxybutyrate (PHB) has been found as the common natural microbial polymer. Various types of biodegradable polymers are under development that popularly includes polylactides, polyglycolic acids, Polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides. On the other hand, natural renewable polymers include porous sponges (from cellulose wood fibers), fibers (made from natural fibers), hydrogels, starch, cellulose, chitin, chitosan, lignin and proteins. Among these numerous mentioned biodegradable polymers, PHAs is being considered as the most potential renewable substitute to petrochemical plastics because of its resemblance to commercially available plastic in the context of physical and chemical properties [10], [11]. Micro-organisms produced PHAs in nutrient deficient/stress condition and utilizes as a carbon source through microbial fermentation [12], [13]. Anderson and Dawes (1990) [14] reported that all PHAs are optically active and consist of (R)-3HA group in their structure. PHA synthase enzyme having stereo specificity which is responsible for R configuration of all PHAs. At the C-3 or  $\beta$ - position, an alkyl group, which can vary from methyl to tridecyl is positioned. This alkyl chain can be saturated, aromatic, unsaturated, halogenated or with branched monomers [15], [16]. Amongst all these PHAs, PHB is of particular interest because they possess a thermoplastic characteristic which is very similar with synthetic plastics and elastomers.

Therefore, replacement of non-biodegradable by biodegradable and eco-friendly polymers like Poly- $\beta$ -hydroxybutyrate (PHB) will help to combat environmental problems created due to the use of synthetic polymers [17]. PHB is a biopolymer which is fully degraded by variety of bacteria [18], fungi and actinomycetes under aerobic and anaerobic conditions in soil, sea, lake, water, sewage etc. After breakdown of PHB some monomers such as  $\beta$ -hydroxy acids, 2-alkenoic acids,  $\beta$ -hydroxyalkanols,  $\beta$ -acyllactones,  $\beta$ -amino acid, and  $\beta$ -hydroxyacid esters are produced. Such monomers are also in various applications as biodegradable solvents [19]. As PHB is biodegradable

and biocompatible, it is used in medical sectors in replacement surgeries. One advantage using such biodegradable material in medical field is that there is no need for surgical removal. Sodian et al. (2000) [20] reported the use of such biopolymers in the preparation of biodegradable heart valve scaffold. Beside this, in bone fracture fixation [21], manufacture of surgical pins, sutures, swabs [22] it is used. Lee 1996 [12] stated that PHAs can be used as carriers for long term dosage of drugs inside the body, insecticides, herbicides and fertilizers. Here again no need for removal of such biodegradable material.

#### 4 PHB PRODUCING MICROBES

Lemoigne in 1925 [23] first demonstrated PHB in *Bacillus megaterium* cells. Since then a wide variety of Gram-positive and Gram-negative organisms are investigated which accumulate PHB as intracellular granules in a highly reduced and insoluble polymer state [24]. Gram positive organisms which produce PHB are: *Bacillus* [25], [26], [27], *Streptococcus* [28], *Rhodococcus* [29]. Many Gram negative organisms such as *Comamonas* [30], *Methylobacterium* [31], *Pseudomonas* [32], *Vibrio* [33], *E. Coli* [34], *Alcaligenes* [35], [36]. These polymers are accumulated under nitrogen stress condition.

#### 5 SCREENING FOR PHB PRODUCTION

For screening of PHB producing positive micro-organisms, different staining techniques are very useful for the detection of PHB into the cell *in vivo*. PHB inclusion is about 0.2 to 0.5  $\mu\text{m}$  in diameter into cytoplasm and can be viewed microscopically [37]. The viable colony staining technique has been suggested as a method for rapid screening of PHB accumulating bacteria. As positive isolates reflects blue color when Sudan Black B ethanolic solution spread over the colonies. Nile blue A fluorescence staining method is also investigated for the detection of PHB inside the cell [38]. Spiekermann et al (1999) [39] reported the viable colony staining technique. This is the direct screening method and it is more sensitive and rapid technique. When PHB positive isolate stain with Nile blue A and observed under UV light it produces strong fluorescence. The PHA producers exhibited strong fluorescence when observed under UV light. Bonartseva et al (2007) [40] stated the use of phosphine 3R stain for the primary qualitative screening of PHB producers. Positive strains produce bright-green fluorescence under UV light.

#### 6 MICROBIAL PRODUCTION OF PHB

While selecting a potential PHB producing strain it is consider that it can grow on renewable substrate such as

vegetable oils, agro waste, industrial waste etc and it can accumulate high PHB content into the cell. Different factors considered while selecting a potential PHB producers such as growth rate of organisms and its rate of synthesis of biopolymer [12]. PHB production in various strains of *Bacillus* has been found in range, from 1.06–41.67% (w/v). The highest storage of PHB i.e. 41.67% w/v in *B. brevis* M6 also reported. *Rahnella aquatilis* and *Stenotrophomonas maltophilia* are the two novel species which is recently reported as they accumulate PHB. The higher PHB accumulation in these organisms is claimed due to the limitation/imbalance of essential nutrients such as nitrogen, phosphate, sulfate, potassium, iron, magnesium, lower oxygen level and C:N ratio of the substrate is higher [41].

Sayyed and Chincholkar (2004) [35] have reported maximum accumulation of PHB in *Alcaligenes faecalis* under nitrogen deficient conditions in two step cultivation process. First step is cell growth in carbon rich medium (CRM) at 30°C at 120 rpm for 24 h. The second step is nutrient deficient copolymer accumulating stage, in which the cell mass obtained after centrifugation of CRM grown in nutrient deficient minimal medium (NDMM) and incubated at 30°C for 48 h at 120 rpm [35]. Excess of carbon sources is required for efficient synthesis of PHB (Lee 1996). Lee (1996) [12] and Haywood et al (1991) [42] reported accumulation of PHB in *Pseudomonas olovarans* from carbon source containing 6–14 C atoms. Fluorescent pseudomonads have been reported to utilize organic acids like acetic acid and lactic acid effectively for the accumulation of PHB [43]. Although, PHB accumulation was evident in pH range, 5.5–9, the level increased towards neutral pH with an abrupt decline beyond 8. Maximum PHB levels have also been reported around neutral pH [44]. Rapske (1962) [45] observed that optimum pH for growth and PHB production by *A. eutrophus* was 6.9. The PHB accumulation is directly proportional to the Carbon: Nitrogen ratio. The hydroxyl acid monomer units depend on the carbon source utilized. Bacteria such as *Alcaligenes eutrophus* utilize various C4 and C5 sources to produce polymers with monomer compositions of 3HB, 4HB, 3-hydroxyvalerate (HV), and 5HV [14]. The C1-C9 alcohols and C2-C10 monocarboxylic acid have also been tested as nutrient sources and proceed useful for PHB production and found that PHB could be obtained with the odd number of carbon sources. Moreover PHB can be synthesized using renewable carbon substrate such as sugars, plant oils, vegetables oils etc. Various waste materials such as whey [46], molasses [47] and starch [48] also used as a carbon source for the production of PHB. As it reduces the production cost and hence useful step in commercialization of such biopolymers.

## 6.1 PHB production by yeast

For the production of PHB various types of yeasts such as *Saccharomyces cerevisiae*, *Candida krusei*, *Kloeckera apiculata* and *Kluyveromyces africanus* also investigated [49]. Moreover the effect of different carbon source and nitrogen source were evaluated. Among them mannitol as a carbon source responsible for highest PHB production upto 21.95%.

## 6.2 PHB production by recombinant bacteria

To enhance PHB accumulation and to produce novel PHB in particular strain, metabolic engineering is best tool to modify the metabolic pathways. PHB biosynthesis genes of *Alcaligenes faecalis* was transferred in *E. coli* strains for the highest accumulation of PHB. The effect of different carbon source (mannitol, glucose, sucrose, lactose, xylose) nitrogen source also studied. To reduce the production cost different cheap substrate as a carbon source such as agricultural waste, whey, cane molasses, vegetable oils etc used in this study. *E. coli* was purposefully chosen as it itself incapable of producing PHB and absence of degrading mechanism of biopolymer. However the growth rate of *E. coli* is faster than other micro-organisms and grows in higher temperature also. The lysis of *E. coli* cell comparatively easy hence helpful in recovery process of PHB. The easy lysis of the cell walls save the cost and time of the purification of PHB granules. Hence, *E. coli* has been selected for transfer of PHB synthesizing genes [32].

## 7 BIOSYNTHESIS OF PHB IN THE CELL

In case of normal metabolism and cell growth, organisms convert glucose into two pyruvate molecules via the glycolytic pathway. Acetyl coenzyme A is synthesized from this pyruvate, which then enters the citric acid cycle, releasing energy in the form of ATP, and GTP as well as NADH which then enters the electron transport chain and donates its electrons to O<sub>2</sub>, where the energy released is trapped in the form of ATP. The PHB synthesis involves the conversion of acetyl-CoA to PHB as a mechanism for storing carbon. The P(3HB) biosynthesis, is the three step biosynthesis pathway consisting of three enzymatic reactions catalyzed by three distinct enzymes as  $\beta$ -ketothiolase, acetoacetyl-CoA reductase and PHB synthase. On the basis of number of carbon atoms in the monomer units as short chain length 3-5 carbon atom and medium chain 6-14 carbon atom. For the synthesis of short chain length PHAs, two acetyl CoA molecules are coupled to form acetoacetyl CoA and a condensation reaction catalyzed by  $\beta$ -ketothiolase by most of the bacteria. While the synthesis of medium chain PHAs undergone into three steps: firstly chain elongation, secondly  $\beta$ -oxidation and third *de novo* biosynthesis using simple compounds [50]. In microbial PHB, the ester bond is formed between carboxyl groups of

one monomer with the hydroxyl group of the neighboring monomer. The PHB is active and isotactic due to the (R) stereochemical configuration [51].

PHB production starts in response to carbon rich and nitrogen limitation condition. Under these conditions (PHB accumulation phase), the cells do not grow or divide normally, instead of that cells shifted their metabolites towards the biosynthesis of hydroxyalkyl-CoA (HA-coA). Further PHB synthase polymerized HA-CoA monomeric precursor to form PHB polyester. PHB form amorphous, white and nearly spherical granules that gradually fill the cells and force them to expand [14] and being insoluble in water.

## 8 RECOVERY OF PHB- SOLVENT EXTRACTION

PHB is soluble in organic solvent; this property leads to the effective and higher PHB extraction from cells. Several solvents such as chloroform, dichloroethane, trichloroethane, ethylene, hexane, propanol, and acetone:alcohol has been in use for isolation and purification of PHB. The treatment of methanol or acetone to biomass, before solvent extraction, denature low molecular weight proteins which enhance the purity of PHB.

Williamson and Wilkinson (1952) [52] first used sodium hypochlorite to isolate PHB granules from *Bacillus cereus*. Sodium hypochlorite resulted in digestion of non-PHB cell mass (NPCM) without affecting the PHB. But sodium hypochlorite purification method was found to affect the sudanophilic properties and molecular weight of the polymer granules. The dispersion of chloroform and sodium hypochlorite is also used for the extraction of PHB. This method slightly reduced the degradation of PHB, but it was highly inconvenient for analytical purposes, which leads to improper data analysis. Extraction of PHB with a mixture of hexane and propanol resulted in poor recovery yield. The solvent system consisting of 1:1 mixture of acetone and alcohol is known to be specific for efficient recovery method capable of specifically lysing the NPCM without affecting PHB [36]. Rawte and Mavikurve (1999) [53] have also claimed the usefulness of acetone and ethanol (1:1 v/v) in the extraction of PHB and reported that ethanol and acetone is the best recovery method giving high recovery yields. PHB recovery by solvent extraction yields a pure white and highly crystalline powder of high molecular weight. Despite of many advantages an inconvenience of solvent extraction methods is that large volume of solvents and non-solvents are needed to extract and to precipitate the polymer.

## 9 BIODEGRADATION OF PHB

Besides the typical polymeric properties, an important characteristic of PHB is their biodegradability (Fig. 1) [54]. Varieties of micro-organisms in nature are able to degrade PHB under the influence of PHB depolymerases [55]. The degradation rate of PHB is typically in the order of a few months (in anaerobic sewage) to years (in seawater) [13]. *Ps. oleovorans* and *Ps. aeruginosa* are also reported to have PHA depolymerases which are probably intracellular depolymerases [56]. Biodegradation of PHA under aerobic conditions results in the formation of CO<sub>2</sub> and H<sub>2</sub>O whereas in anaerobic conditions, the degradation products are CO<sub>2</sub> and CH<sub>4</sub> [57]. P(HB-HV) is biodegraded in microbial active environments. Microorganisms colonize on the surface of the polymer and secrete hydrolyzing enzymes which degrade P(HB-HV) into HB and HV units. These monomeric units are utilized by the cells as a carbon source for biomass growth [12].

Kim et al (1997) [58] observed that fungal biomass in soils generally exceeds the bacterial biomass and thus it is likely that fungi may play a considerably significant action to degrade polyesters in the soil ecosystem. However the microbiological and environmental aspects of fungal biodegradation of polyesters are not yet known. Lee et al. (1994) [59] studied the degradation of PHB by fungi from samples collected from various environments. PHB depolymerization was tested in plates containing PHB as the sole carbon source in medium and spot inoculated with fungal isolates from the samples. The biodegradation activity was investigated by observing the hydrolyzing zone around the colony. Romen et al (2004) [60] isolated 18 Gram-negative thermotolerant PHB degrading bacteria from compost. These isolates produced hydrolysis zones on PHB agar plates, indicating the presence of extracellular PHB depolymerases.

Extracellular degradation of PHB is the utilization of an exogenous polymer by not a necessarily accumulating microorganism that secretes extracellular PHA depolymerase. Many extracellular PHB depolymerases have been isolated and their mode of action has been studied [61]. These extracellular depolymerases get adsorbed on to the insoluble PHB first and then hydrolyze the polymer chains. Enzymatic hydrolysis of P(3HB) results in 3HB dimer as the major product besides small amounts of 3HB monomer. These cleave mainly the second and third ester linkages from the hydroxyl terminus [11].

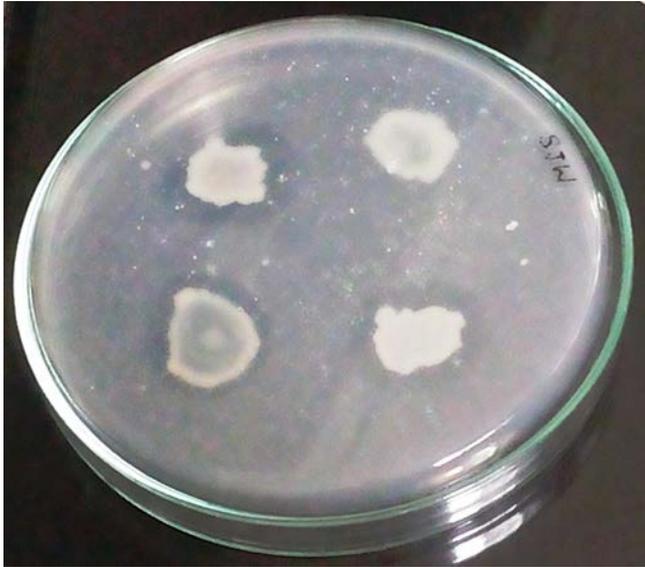


Fig. 1 Biodegradation of PHB

### 9.1 Physico-chemical factors affecting biodegradability

The rate of polymer degradation depends on a variety of factors including surface area, microbial activity of the disposal environment, pH, temperature, moisture and presence of other nutrient materials. P(HB HV) is water insoluble and is not affected by moisture [61]. The effect of different environments on the degradation rate of PHB and P(HB-HV) has been studied by several workers [62]. It was stated that most of poly (HA)-hydrolyzing enzymes differ highly in their substrate specificities for various poly(HA). Polymeric materials have specific and accurate structural behavior which expresses their physical integrity and property. A slight variation in the physical factor will result into large interfere in biodegradability. On the other hand, the specificity, stereo regularity, crystallinity and composition of poly(HA) have a noticeable effect on the biodegradability of polymer [63], [64], [65]. In terms of crystallinity of the polymer, the degradability of a polyester decreases with increase in its crystallinity [66], [67]. Molecular weight [Mw] is one of the factors determining the biodegradation of plastics. A higher molecular weight of polymer lowers the biodegradation by the microorganisms because its high molecular weight makes it unfavorable and inaccessible for microbial attack due to decrease in the solubility of polymer and hence low molecular weight is favorable for biodegradation of biopolymer. The melting temperature [Tm] of a polymer also greatly affect on enzymatic degradability of PHB. UV light can accelerate the degradation of PHAs [68]. Ying et al (2006) [69] described the application of UV radiation on PHB degradation and reported significant reduction in

the Mw, such molecular weight loss was dependent on the time exposure of UV radiation. Under direct UV radiation the film of PHB became very brittle. In comparison to their degradation was faster than that of the powder subjected to direct UV radiation. Zhang et al. (1994) [70] has reported that copolymers with low MW and higher purity can be obtained in a short time after degradation. Ultrasonic radiation is not fully involved in biodegradation, it only helps and promotes the biodegradation of PHB.

## 10 COMMERCIAL APPLICATION OF PHB

### 10.1 Medical and pharmaceutical applications

Medical implant is one of the fastest growing areas in the last few years. Hence it is essential to use the biocompatible material for successful surgeries of implantation. The monomers forms after biodegradation of PHB is the intermediate in metabolic pathways of all higher organisms [12], [41]. Hence it is considered to be biocompatible in all higher organisms without any toxicity. Other application includes the use of biodegradable polymer for site specific drug delivery, surgical pins, sutures, and swabs, wound dressing, bone replacements and plates, blood vessel replacements, stimulation of bone growth and healing by piezoelectric properties, cardiovascular, dental implants, biodegradable matrix for drug release in veterinary medicine etc. The advantage of using biodegradable plastics during implantation is that it will be biodegraded, i.e., no need for surgical removal. Wang and Bakken (1998) [71] reported that particularly PHB could be used as biodegradable carriers for long term dosage of drugs, medicines and hormones. Brandl et al. (1988) [72] considered PHB as a source for the synthesis of chiral compounds (enantiomerically pure chemicals) and also it is used as raw materials for the production of paints. PHB could be depolymerized to rich source of optically active pure bifunctional acids. It is also used in the synthesis of beta lactam group of antibiotics.

### 10.2 Agricultural applications

As PHB is degraded in soil hence their use in agriculture is of priority interest. It can be used as biodegradable carriers for long-term dosage of insecticides, herbicides, or fertilizers, seedling containers and plastic sheaths protecting samplings as tubing for crop irrigation. Here again, it is not necessary to remove biodegradable items at the end of the harvesting season [41].

### 10.3 Food commodity packaging applications

As PHB having some similar properties to synthetic plastic such as tensile strength and flexibility, it is used in food packaging [41]. PHB is used in packaging material such as bags, containers and paper coatings. Other applications as conventional commodity plastics

include the disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetic containers, shampoo bottles and cups. Oeding and Schlegel (1973) [73] reported that PHA has wide range of applications owing to novel features.

#### 10.4 Disposable personal hygiene

PHB can be used as sole structural materials or as parts of degradable plastics.

#### 10.5 Tissue engineering scaffolds

The suitable material properties of PHB such as biocompatibility, support cell growth, guide and organize cells allow tissue in growth makes it an ideal candidate for tissue engineering scaffolds.

#### FUTURE PERSPECTIVE

Isolation of PHB synthesizing genes from potent PHB producer to transform these genes into easily cultivable organisms, for greater accumulation of PHB. Recombinant *E. coli* used to produce PHB under varying cultural conditions.

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