

# Optimization on Microbial Production of Polyhydroxybutyrate (PHB): A Review

Punniavan Sakthiselvan<sup>1\*</sup> & Ramasamy Madhumathi<sup>2</sup>

<sup>1\*</sup> Assistant Professor, Department of Bio-Engineering, School of Engineering, VELS Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai 600 117, Tamil Nadu, India.

<sup>2</sup> Department of Chemical Engineering, A.C. College of Technology, Anna University, Chennai 600 025, Tamil Nadu, India.

Received: September 13, 2018

Accepted: October 30, 2018

**ABSTRACT:** Plastic is considered to be an individual gift of modern science and technology to mankind. This unique wonder material has some mutually exclusive qualities of being very light, yet strong and economical. The mammoth scale of the use of plastics and their disposal has threatened natural environment. Nowadays, plastics and synthetic polymers are mainly produced from petro-chemical elements, which do not decompose, thus resulting in the environmental pollution. They are stored, burnt and recycled. During combustion, water and carbon dioxide are released into the atmosphere. In household waste, 30 percent are plastic- packaging foil, like food bags or coated foil on paper. The reorganization of environmental pollution problem caused by synthetic plastics has led to the search of alternative materials- the biodegradable plastics. Biodegradable plastics of renewable resources origin also help to preserve the non-renewable resources and contribute to sustainable development. In recent years, there has been an increasing trend towards more efficient utilization of relatively cheaper substrates for Polyhydroxybutyrate (PHB) biosynthesis (via) formation. Finding a less expensive substrate is, therefore, a major need for a wide commercialization of PHB. Production processes based on waste carbon sources are the requirement of the day, instead of hobbler one. In biotechnological aspects, cheap substrate and genetically modified high PHB yielding bacteria or plants can be used in the biopolymer production technique.

**Key Words:** Environmental pollution, Biopolymer, Polyhydroxybutyrate, Microbes, Plants.

## INTRODUCTION

In recent years, sustainability, environmental concerns and green chemistry have played a big role in guiding the development of the next generation of materials, products and processes. The persistence of plastics in the environment, dwindling petroleum resources, shortage of landfill space and the concerns over emissions of toxic gases during incineration have fuelled efforts to develop biodegradable polymers from renewable resources [1]. Particularly, renewable agricultural and biomass feedstock have shown much promise for use in eco-efficient packaging to replace petroleum feedstock without competing with food crops.

Polyhydroxyalkanoates (PHAs) is natural and linear polyesteric biological macromolecules that can be produced by several microorganisms in the form of intracellular carbon and energy storage granules in response to excess carbon substrate and limited quantities of nitrogen source in growth environment. They are also completely degraded into carbon dioxide and water by the depolymerises present in the microorganisms [2]. Polyhydroxybutyrate (PHB) was the first type of PHAs discovered and the most widely studied. PHB share many material properties similar to synthetic polymers, along with exclusive properties such as biodegradable, biocompatible and can be obtained from renewable sources, but its production costs are higher than the petroleum derived plastics [3].

## ISOLATION, DETECTION AND ANALYSIS

### Microorganisms that Produce PHA

A wide variety of bacterial species are known to accumulate PHA [4]. PHA has been reported from various environments such as soil, sewage sludge, marine sediments, ponds, mangrove environments [5] and Gas field soil [6]. Microorganisms that produce PHA are discussed with respect to two aspects viz. microbiological and basic genetic makeup of the organism and the economic aspects for PHA production [7].

### Oxygen limitation

During oxygen limitation nicotinamide nucleotides are not oxidized. This will result in decrease of effectiveness of TCA cycle and activity of citrate synthase and isocitrate dehydrogenase is decreased by NADH. Thus acetyl-Co A accumulates and there is a low intracellular concentration of free CoA. Increase in

acetyl-CoA/CoASH ratio partially relieves the inhibition on  $\beta$ -ketothiolases and favours PHB formation.

### **Phosphorus and magnesium limitation**

During phosphorus and magnesium limitation, PHA accumulates from the beginning of the cell growth. Magnesium ions are known to increase the growth rate and phosphorus is an essential nutrient for the living organism, which is required for the regulation of the physiological state and energy metabolism. It would not be possible to achieve high mass and PHB content at low levels of phosphorus. As phosphate does not participate directly in protein composition, its limitation will allow residual cell growth for some time [8]. Phosphorus is involved in recycling of energetic intermediates i.e. ATP /ADP. In its deficiency, there is a demand from NADP and hence will lead to PHA production.

### **Nitrogen limitation**

If nitrogen is limiting then acetyl-CoA and NAD (P) H are restricted. Liberated NAD (P) H cannot be consumed for reductive synthases, i.e. for amino acid synthesis. As there is absence of protein synthesis, acetyl-CoA will be channeled into PHB synthesis. For effective synthesis of PHA, Acetyl-CoA and NADPH are necessary. The intracellular concentration of acetyl-CoA should be high. The flux of acetyl- CoA either towards the TCA cycle for cell growth or PHB biosynthesis pathway for energy storage has been controlled by NADPH/NADP ratio. An elevated level of NADPH significantly enhances PHB accumulation [9].

### **PHB production by bacteria**

The isolation of PHA producing bacteria and characterized the potent PHA producer as *Sphingomonas* sp. as confirmed by 16s rDNA sequencing. In their study, the strain was grown on different sugars and organic acids and its ability to accumulate PHA was analyzed. The strain could accumulate PHA when grown on disaccharides, aldohexose, sugaralcohols and some organic acids, but failed to assimilate ketoses, pentoses and starch. Among the sugars tested, PHA yield was found to be high with sucrose or mannose, contributing to 55-60 % of cell biomass. Pal & Paul (2000)[10] isolated aerobic free-living nitrogen fixing bacteria from natural environments. Systematic screening of these isolates has indicated that nearly 70 % of isolates of the genus *Azotobacter* were capable of accumulating PHB. The PHB contents of majority of the strains ranged from 25-47 % of cell dry weight, while only 7 isolates accumulated PHB accounting to more than 50 % of their cell dry weight. One of the promising strains of *Azotobacter chroococcum* has been shown to accumulate the polymer accounting nearly to 70 % of cell dry weight, when grown under optimized conditions. A gram-positive bacterium (designated as strain no INT005) isolated from a field soil that accumulated polyhydroxyalkanoate (PHA) from a grass field soil. The PHA productivities of strain INT005 were higher than those of *Bacillus megaterium* and *Ralstonia eutropha* at 37-45 °C and the PHA exhibited moderate thermostability. The potentialities of phototropic purple non sulphur bacteria for PHB synthesis [6]. They isolated 30 organisms from water and sludge samples collected from different water bodies of West Bengal by enriching under phototropic microaerophilic conditions. Systematic screening of these isolates for PHB production led to the identification of five strains with PHB content ranging from 10-15 % of cell dry weight, when grown in acetate containing medium under a light intensity of 10000 lux. Carbon sources like acetate and butyrate were most suitable for PHB accumulation. However, the presence of nitrogen source in the growth medium was found to be inhibitory for PHB accumulation although the growth was enhanced. Phosphate and sulphate limiting conditions enhanced the polymer accumulation by the isolates. They also evaluated the effects of physical factors like pH, light intensity on polymer accumulation. *Pseudomonas* sp. 14-3, a strain isolated from Antarctic environments that accumulated large quantities of polyhydroxybutyrate (PHB) when grown on octanoate. This isolate was characterized on the basis of phenotypic features and partial sequencing of its 16s ribosomal RNA gene [11]. PHB producing bacteria isolated from different locations such as garden soil, tannery effluents, sewage sludge and field soil. They obtained higher PHB positive strains from sewage sludge and tannery effluents compared to other sources [12]. Isolation of 29 *Bacillus* strains from the grassland soils of Ankara, Turkey and were identified as *B. brevis*, *B. sphaericus*, *B. cereus*, *B. megaterium*, *B. circulans*, *B. subtilis*, *B. licheniformis* and *B. coagulans*. Polyhydroxybutyrate (PHB) production by these strains was determined by the spectrophotometric method, and they found that PHB production ranged from 1.06–41.67 % (w/v) depending on the dry cell dry weight. The highest PHB production and productivity percentage was found in *B. brevis* M6 (41.67 % w/v) [13].

### **PHB production by cyanobacteria**

*Synechocystis* pcc6803 accumulated the PHA content was about 5 % of cell dry weight. They showed that the biosynthesis could be improved by introducing multicopies of heterologous pha synthase gene. Nile blue A staining and freeze fracture electron microscopy revealed the presence of many PHA inclusions in the cell cytoplasm. The relatively low weight of PHA in the cyanobacterium when compared to other bacteria

was probably due to the small size and mass. They also reported that PHA synthesizing ability of the cyanobacterium might, in fact, be quite similar to that was shown by most bacteria in nature [14].

### **PHB production by yeasts**

An engineering of novel pathways seems to be a beneficial a stiff crystalline alternative to the production of PHBs in yeast cells were used as models to gain information on PHBs synthesis in eukaryotes. Yeasts as hosts for synthesis of PHBs have certain advantages over bacteria. Moreover, yeast like *Saccharomyces cerevisiae*, *Kluyveromyces fragilis*, *Candida utilis* and others, has been approved as a GRAS microorganisms by food and drug administration. PHB homopolymer production in two transgenic yeasts One of them (*Saccharomyces cerevisiae* INVSc1/PHA1) harbouring the PHB synthase genes of *R. eutrophus* in its cytoplasm, while in the second (*Schizosaccharomyces pombe* Q01/PHB), PHB biosynthesis genes were integrated into the chromosome [14].

### **PHA production by recombinant bacteria**

PHAs are commonly grouped into two major categories: the short-chain-length PHAs (PHASCL) and the medium-chain-length PHAs (PHAMCL). The repeat units of PHASCL are composed of hydroxy fatty acids having three to five carbon atoms, whereas PHAMCL contain hydroxy fatty acids repeat units with six or more carbon atoms. Because of their biodegradable and biocompatible properties, PHAs have been extensively researched as potential “green” substitutes for petroleum-derived polymers in medicine, drug-delivery, agriculture and horticulture, the fibers industry, and consumer products [15]. *Escherichia coli*, as the best-known bacteria, is an ideal host for the production of PHAs. It is suitable as a heterologous expression host for foreign genes that can be easily manipulated and improved by means of recombinant DNA methodologies or metabolic engineering. In addition, high-cell-density cultivation strategies for numerous *E. coli* strains are well. Metabolic pathways of PHAs in *E. coli*, including PHASCL and PHAMCL, have already been setup since 10 years ago established.

*E. coli* cells that accumulate large amounts of PHASCL become fragile, facilitating the isolation and purification of the biopolymer. Furthermore, the bacterium does not express PHA-degrading enzymes [16]. The production of PHAs employing recombinant *E. coli* was restrained in both laboratory scale and industrial scale due to low efficiency and high cost. In polyhydroxybutyric acid (PHB) production, about 40 % of the total production cost is for raw material [17]. Thus, the use of a cheaper carbon source is required to reduce the high production cost of PHAs, which have been successfully used in various wild strains [18]. Agriculture and its associated industries can be the major pool of these cheap carbon sources because they produce many feedstocks and co-products that are attractive raw materials for microbial production of PHAs, such as whey, ice cream residue, etc. From an ecological point of view, they are renewable, whereas from an economic point of view, many of the co-products being studied are derived from surplus or low-cost processing streams [19]. Metabolic engineering is being intensely explored to introduce new metabolic pathways to broaden the utilizable substrate range, to enhance PHA synthesis and to produce novel PHA. Recombinant *E. coli* strains harbouring the *Alcaligenes eutrophus* PHA biosynthesis genes in a stable high-copy-number plasmid have been developed and used for high productivity. Since *E. coli* can utilize various carbon sources, including glucose, sucrose, lactose and xylose, a further cost reduction in PHA is possible by using cheaper substrates such as molasses, whey and hemicellulose hydrolysate. Natural PHA-producing bacteria have a long generation time and relatively low optimum growth temperature. These are often hard to lyse and also contain pathways for PHA degradation. Bacteria such as *E. coli* are incapable of synthesizing or degrading PHA; however, *E. coli* grows fast, even at higher temperature and is easy to lyse. Faster growth will enable it to accumulate a large amount of polymer. The easy lysis of the cells save the cost of the purification of PHA granules [20]. *E. coli* has been used to transfer PHA genes. PHB production has been studied mostly in recombinant *E. coli* cells harbouring PHA synthesizing genes from *R. eutropha* [21].

### **PHA production in transgenic plants**

Currently, PHAs for commercial applications are being produced by microbial fermentations. However, continuing efforts are being made to devise cost-effective means such as transgenic plants, to produce PHAs. Transgenic plants have always been utilized for producing genetically modified (GM) crops for food purposes and have always attracted suspicion and ethical debates. Producing PHAs in plants, on the other hand, will help connect the low cost/high-volume sustainable production capacity of crops with the vast number of developing polymer industries and should not promote much controversy. This will enable PHAs to possess both superior cost and performance factors. Many research groups have reported their attempts to produce PHAs in plant systems. The only raw materials required will be carbon dioxide for carbon and sunlight as the energy source. The overall costs would make PHA production economical. Transgenic plants containing the PHA synthase genes have been created. The transgenic plants were stunted

but accumulated about 15 % dcw (dry cell weight) of P (3HB) in the leaf expression systems. Co-polymers of 3HB and 3HV were produced in *Arabidopsis* and *Brassica rapa*. A molecular mass of was attained which is excellent for commercial applications. The production of PHAs in plastids whereas Poirier's group targeted PHA production in seeds. *Arabidopsis* plants accumulated P (3HB) up to 14 % of the leaf dry weight. Transgenic PHA-producing plants have also been produced using tobacco, cotton, and flax systems. However, plant systems have other disadvantages. It is difficult to produce copolymers in plant systems since the production is under the control of endogenous metabolic precursors in the plant. Similarly, the recovery of the polymer from plant tissues is a tricky and expensive affair. Researchers in Japan have also recently devised a transgenic *Arabidopsis thaliana*, harbouring an engineered PHA synthase gene from *Pseudomonas* sp. 3; *fabH* gene (codes for 3-ketoacyl-ACP synthase III) from *E. coli* and a *phaAB* gene (codes for a ketothiolase and acetoacetyl-CoA reductase) from *Cupriavidus necator* and the enzymes were targeted to the plastids. A polymer consisting of 3-hydroxybutyrate unit and a small portion of 3-HA units (C5–C14) was produced. The *fabH* gene aided a two-fold increase in the average PHA content but the maximum PHA amount remained the same and any further increase led to stunted growth. However, the tissue-specific PHA production countered that. The same group also carried out seed-specific PHA production in rice and tuber-specific production in potato. Metabolix in alliance with British Petroleum has now successfully transferred the PHA metabolic pathway into switchgrass (*Panicum virgatum*). Switchgrass grows quickly, converting solar energy into chemical energy. Switchgrass also absorbs carbon dioxide from the atmosphere as it grows, thus reducing the build up of this gas in the atmosphere. They have developed a detailed biorefinery cost and engineering analysis using switchgrass, which is being used to promote large-scale PHA manufacturing. Hence, altered plant phenotypes, low productivity and transgenic stability are problems that have to be resolved before transgenic plants become the chosen mode of PHA production [22].

#### **Screening methods for PHB accumulation in bacteria**

Native PHA granules can be stained with Sudan Black B [23], Nile Blue A and Nile Red. PHAs are more specifically stained by Nile blue A, where its presence is indicated by strong orange fluorescence. The structure, physio-chemical properties, monomer composition and the number and size of the PHA granules vary depending on the organism. GC-MS spectra of the PHA accumulated by various isolates as well as PHA standards confirmed that the polymer produced is mainly PHB except in case of VK-9 in which an additional peak at 5.27 min [24]. Transmission electron microscopy (TEM) and Atomic Force Microscopy (AFM) have revealed early granule formation in *Wausteria eutropha* H16. Earlier electron microscopy studies of PHB granules from *Bacillus megaterium*, *Chlorogloeafritschii* focused on various models leading to formation of PHB granules, but no work has been reported on simultaneous qualitative and quantitative estimation of intracellular PHB granule synthesis in *Alcaligenes* sp [25]. PHAs can be observed under phase contrast light microscope, as discrete granules sized between 0.2 to 0.5  $\mu\text{m}$  in diameter which is localized in the cell cytoplasm. Under transmission electron microscope (TEM), it appears as electron-densed bodies. In terms of molecular weight, PHA can weigh between  $2 \times 10^5$  to  $3 \times 10^6$  Daltons [26]. PHB granules isolated from the *B. thuringiensis* cells observed under scanning electron microscope showed stable spherical configuration with an average diameter of 5 microns. The NMR spectra identified the polymer as an isolatic homopolymer. The spectrum revealed the presence of three groups of signals characteristics of PHB homopolymer. The doublet at 1.3 ppm was attributed to the methyl group coupled to one proton, the doublet of the quadrant at 2.57 ppm to the methylene group adjacent to an asymmetric carbon atom bearing a single proton and the multiplet at 5.28 ppm to the methylene group, chloroform gave a chemical shift signal at 7.25 ppm. FTIR analysis revealed two absorption bands at  $1280\text{cm}^{-1}$  and  $1735\text{cm}^{-1}$  corresponding to C=O and C-O stretching groups respectively. The gel permeation chromatography (GPC) analysis of the polymer isolated from *B. thuringiensis* R1 cells revealed that the polydispersity index(Q) [27].

#### **PCR techniques for detection of PHB producers**

Colony PCR and semi-nested PCR techniques for screening polyhydroxyalkanoate (PHA) producers, that was isolated from different environments. Three degenerate primers were designed based on multiple sequence alignment results and were used as PCR primers to detect PHA synthase genes. The sensitivity limit of the colony PCR was  $1 \times 10^5$  viable cells for *Ralstonia eutropha*. The results have suggested the application of this PCR protocol for rapid detection of PHA producers from the environment. The colony PCR to detect the PHA synthase genes from bacterial strains isolated from various ecosystems. For this, they isolated the genomic DNA used as a template. Colony PCR was performed following the procedure of [28]. In each PCR analysis, 30 mg of genomic DNA of Nile red and Nile blue A positive strains and three degenerate primers phacF1, phacF2 and pha-CR4 were used to detect PHA synthase genes. PCR amplified DNA fragments were observed by agarose gel electrophoresis in 1 per cent agarose gel. The amplified DNA fragments were visualized by UV illumination.

### Substrates and Growth Conditions for PHB Production

The production of PHB by *Methylobacterium extorquens* ATCC 55366 using methanol as the sole carbon and energy source in a fed-batch fermentation system. The production of PHB between 40 % and 46 % on a dry-weight basis was produced by *M. extorquens*. In addition, biomass production and the growth rate of *M. extorquens* were affected by the mineral composition supplied. The absence of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> or MnSO<sub>4</sub> and the absence of a combination of CaCl<sub>2</sub>, FeSO<sub>4</sub>, MnSO<sub>4</sub> and ZnSO<sub>4</sub> had a negative impact on biomass production and the specific growth rate of this bacterial strain. Tested 37 isolates and mutants of *Azotobacter chroococcum* for PHB production using Sudan black B staining method. With 2 % glucose and 15 mM/L ammonium acetate, PHB production was found to be maximum at 36 and 48 hours of growth under submerged cultivation and under stationary cultivation respectively. They also observed that PHB production was higher on sucrose and commercial sugar as compared to glucose and mannitol. Among inorganic nitrogen sources, they found ammonium acetate (15 mM/L) to be the best for PHB production [23]. PHB production from *Methylobacterium* SPV-49. They evaluated different carbon sources. Maximum accumulation of PHB was observed with glucose as the carbon source. Methanol and sugars such as sucrose and lactose also induced PHB accumulation. The effect of C: N ratio on polymer accumulation was studied. An organic nitrogen source increased the PHB accumulation rather than inorganic nitrogen sources based on the study on *Bacillus mycoides*. Higher growth of *R. eutropha* ATCC 17697 and *A. latus* ATCC 29712 using organic nitrogen compared to inorganic nitrogen, while there was high PHB production by the strains in media supplemented with ammonium sulphate as nitrogen source. There are many reports to establish the effect of different nitrogen sources on *Bacillus sp.* [29], *Streptomyces sp.* [30], *Rhizobium sp.*, *A. latus* and *R. eutropha*. The PHA production phase, the cell growth is limited owing to the depletion of essential nutrients such as nitrogen, phosphorus, magnesium, among others. This depletion in the presence of excess carbon source triggers the metabolic shift from growth to PHA production phase. *Cupriavidus necator* (formerly known as *Wautersia eutropha*, *Ralstonia eutropha* and *Alcaligenes eutrophus*) has been used for optimal production of polyhydroxybutyrate PHB, a homopolymer that is accumulated under nitrogen limitation. *Azotobacter beijerinckii* produces PHA under oxygen limitation compared to nitrogen or phosphorus limitation. A wide variety of PHA copolymers are synthesized in *Bacillus sp.* from fermentation of different carbon sources [31].

### Inexpensive Substrates for PHA Production

The production cost of PHB totally depends on the microorganisms, the use of inexpensive substrates, the nutritional and process parameters and the recovery and purification process. Since, carbon source has the major influence over the (PHB) production cost, rummage around for inexpensive carbon sources has become one of the significant research in the PHB production [32]. Agro industrial wastes are attractive candidates since they have some of the desired characteristics namely low prices and high availability. A wide variety of substrates such as whey, lignocellulosic materials and waste water has been used with different microorganisms to improve the yields of PHB production and also to avoid the environmental disposal problems [33]. *Azotobacter chroococcum* MAL-201 accumulated PHB. The polymer yield accounted to 69 % of cell dry weight when grown in N<sub>2</sub>-free stockdale medium containing 2 % (w/w) glucose. To make the polymer production cost effective, 12 wastes of different origin were tested for growth and polymer production. The candy factory waste was found to be the most suitable substrate for homopolymer production. The accumulated PHB accounted 17.8 and 40.58 % of cell dry weight under single step and two step cultivation conditions, respectively [34]. *B. megaterium* was grown on various carbon sources such as date syrup and beet molasses [35]. Best results with regard to growth and PHB production were obtained in these cheaper carbon sources. Khardenaviset al (2003) [36] used activated sludge from a food processing industry and waste water from different industries, as substrates for the production of bioplastics. Amongst the different waste water tried, anaerobic treatment waste water gave maximum production of bioplastics followed by deproteinized milk whey and soy whey; thereby achieving twin objectives of effective utilization of available resources for production of value-added products and reduction in cost by solving the problem of waste disposal.

**Table 1:** PHB (and its copolymers) biosynthesis from inexpensive substrates. The table includes substrate, microorganism and reference

Microorganisms	Substrate	Reference
<i>Ralstonia pickettii</i> 61A6	Sugar cane liquor	[37]
<i>Bacillus cereus</i> M5	Sugar cane and beet molasses	[13]
<i>Pseudomonas fluorescens</i> A2a5	Sugarcane waste	[38]

<i>Azotobacterchroococcum</i> and Recombinant <i>Escherichia coli</i> strain	Starch-based materials	[39]
Recombinant <i>Escherichia coli</i>	Molasses	[40]
<i>Burkholderiacepacia</i> IPT 048 and <i>B. sacchari</i> IPT 101	Cellulose hydrolysates, xylose etc	[41]
Recombinant <i>Escherichia coli</i>	Dairy whey	[42]
<i>Pseudomonas hydrogenovora</i>		[43]
<i>Methylobacterium sp.</i> ZP24		[44]
<i>Bacillus sp.</i> CFR 256	Corn steep liquor	[45]
<i>Bacillus megaterium</i> ATCC 6748	Corn steep liquor and molasses	[46]
<i>Cupriavidusnecator</i>	Corn syrup	[47]
<i>Bacillus safensis</i> EBT1	Sugarcane baggasse	[48]
<i>Cupriavidus</i> sp. USMAA2-4	Crude palm kernel oil	[49]
<i>Ralstoniaeutropha</i>		[50]
<i>Pseudomonas aeruginosa</i>	Fatty acids and waste glycerol	[51]
NCIB 40045		[52]
<i>Cupriavidusnecator</i>		
Paper mill wastewater	Activated sludge	[53]
<i>Azotobactervinelandii</i> UWD	Swine waste liquor	[54]
<i>Pseudomonas putida</i> CA-3	Petrochemical plastic waste	[55]
<i>Pseudomonas hydrogenovora</i>	Sunflower cake, soy bran and olive mill	[43]
Mixed bacteria	Soy and malt wastes	[56]
<i>Bacillus sp.</i>	Waste activated sludge	[57]
<i>Bacillus megaterium</i>	Dairy waste and sea water	[58]
<i>Pseudomonas putida</i> KT2442	Waste water from olive oil mills (called alpechin)	[59]
<i>Azotobacterchroococcum</i> H23		[60]
<i>B. subtilis</i> MSBN17	Tamarind kernel powder	[61]
Recombinant <i>Bacillus subtilis</i> strains	Acid-hydrolysed malt waste	[62]
<i>A. australica</i>	Beet molasses, cane molasses	[63]
	maple sap	[64]
<i>Bacillus sp.</i>	Groundnut oil cake	[65]
<i>Pseudomonas putida</i> Bet001	palm oil mill effluent	[66]

## Conclusions

In the recent years, there has been increasing public concern over the harmful effects of petrochemical-derived plastic materials in the environment. Plastics being xenobiotic are recalcitrant to microbial degradation. Excessive molecular size seems to be mainly responsible for the resistance of these chemicals to biodegradation and their persistence in soil for a long time. Nature's built-in mechanisms and self-regulation ability cannot tackle novel pollutants since these are unfamiliar to it. This has prompted many countries to start developing biodegradable plastic. According to an estimate, more than 100 million tonnes of plastics are produced every year. Several hundred thousand tonnes of plastics are discarded into marine environments every year and accumulate in oceanic regions. Incinerating plastics has been one option in dealing with non-degradable plastics, but other than being expensive it is also dangerous. Harmful chemicals like hydrogen chloride and hydrogen cyanide are released during incineration. Recycling also presents some major disadvantages, as it is difficult sorting the wide variety of plastics and there are also changes in the plastics material such that its further application range is limited. Replacement of non-biodegradable by degradable plastics of major interest both to decision-makers and the plastic industry. Making eco-friendly products such as bioplastics is one such reality that can help us overcome the problem

of pollution caused by non-degradable plastics. Thus, it becomes inevitable for us to improve upon the method of production, selection of raw materials, recycling, conversion to suitable forms of certain wastes, so that we do not add any material waste into the environment which nature cannot take care of.

## REFERENCES

1. Lisa, A. Anderson, M. Ahsanul Islam & Kristala L. J. Prather 2018. Synthetic biology strategies for improving microbial synthesis of “green” biopolymers, Synthetic biology for green biopolymers. <http://www.jbc.org/cgi/doi/10.1074/jbc.TM117.000368>.
2. Suriyamongkol, P, Weselake, R, Naraine, S Moloney, M & Shah, S 2007. Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants-A review, *Biotechnological Advances*. 25:148-175.
3. Bora, L 2013. Polyhydroxybutyrate Accumulation in *Bacillus megaterium* and Optimization of Process Parameters Using Response Surface Methodology, *Journal of Polymer and Environment*, 21:415-420.
4. Hawas, J.M.E, El-Banna, T, Belal, E.B.A & El-Aziz, A.A. 2016. Production of Bioplastic from some selected Bacterial strains', *International Journal of Current Microbiology and Applied Sciences*, 5(1): 10-22.
5. Nubia, M, Ivonne, G, Dionisio M, Victoria, G, Dolly, R, Diego, S, Juan, G, Fabio, A, Armando, E & Dolly, M 2007. Bioprospecting and characterization of poly-beta-hydroxyalkanoate (PHAs) producing bacteria isolated from Colombian sugarcane producing areas, *African Journal of Biotechnology*, 6(13): 1536-1543.
6. Tajima, K, Igari, T, Nishimura, D, Satoh, Y & Muneakata, M 2003. Isolation and characterization of *Bacillus* sp. INT005 accumulating polyhydroxyalkanoate (PHA) from gas field soil, *Journal of Bioscience and Bioengineering*. 95:77-81.
7. Sinha, K & Rathore, P 2015. Study of Polyhydroxybutyrate producing *Bacillus* sp. isolated from Soil, *Research Journal of Recent Sciences*. 4:61-69.
8. Squio, C R, Marangoni, C, De Vecchi, CS, Aragao, GMF 2003. Phosphate feeding strategy during production phase improves poly (3-hydroxybutyrate-co-3-hydroxyvalerate) storage by *Ralstonia eutropha*, *Applied Microbiology and Biotechnology*. 61: 257-260.
9. Choi, JY, Lee, JK, You, Y & Park, WH 2003. Epoxidized Polybutadiene as a Thermal Stabilizer for Poly (3-hydroxybutyrate). II. Thermal Stabilization of Poly (3-hydroxybutyrate) by Epoxidized Polybutadiene, *Fiber Polymer*. 4:195-198.
10. Pal, S & Paul, AK 2000. Accumulation of biodegradable plastics by *Azotobacter*. In: 59th Annual Conference of Association of Microbiologists of India, pp.58.
11. Ayub, ND, Pettinari, MJ, Ruiz, JA & Lopez, NJ 2004. A polyhydroxybutyrate producing *Pseudomonas* sp. isolated from Antarctic environments with high stress resistance, *Current Microbiology*. 49: 170-174.
12. Sujatha, K, Mahalakshmi, A & Shenbagarathai 2005. A study on accumulation of PHB in native *pseudomonas* isolates LDC-5 and LDC-25, *Indian Journal of Biotechnology*. 4:216-221.
13. Yilmaz, M, Soran, H & Beyatli, Y 2005. Determination of Poly-β- hydroxybutyrate (PHB) production by some *Bacillus* sp., *World Journal of Microbiology and Biotechnology*. 21: 565-566.
14. Erich Markl, Hannes Grünbichler & Maximilian Lackner 2018. PHB - Bio Based and Biodegradable Replacement for PP: A Review Novel Techniques in Nutrition and Food Science, 2(4): 1-4.
15. N. A. Pattanashetti, Geetha B. Heggannavar, Mahadevappa Y. Kariduraganavar 2017. Smart Biopolymers and Their Biomedical Applications *Procedia Manufacturing* 12:263 – 279.
16. Aldor, IS & Keasling, JD 2003. Process design for microbial plastic factories: metabolic engineering of polyhydroxyalkanoates', *Current Opinion in Biotechnology*. 14:475-483.
17. Huang, TY, Duan, KJ & Huang, SY 2006. Production of polyhydroxyalkanoates from inexpensive extruded rice bran and starch by *Haloferax mediterranei*, *Journal of Microbiology and Biotechnology*. 33:701-706.
18. Solaiman, DKY, Ashby, RD, Foglia, TA & Marmer, WN 2006. Conversion of agricultural feedstock and coproducts into poly (hydroxyalkanoates)', *Applied Microbiology and Biotechnology*. 71: 783-789.
19. Keenan, TM, Tanenbaum, SW & Nakas, JP 2006, 'Microbial formation of polyhydroxyalkanoates from forestry-based substrates', *ACS Symposium Series*. 921:193-209.
20. Li, R, Zhang, HX & Qi, QS 2007, 'The production of polyhydroxyalkanoates in recombinant *Escherichia coli*', *Bioresource Technology*. vol. 98, no.12, pp. 2313-2320.
21. Sayeed, RZ & Gangurde, NS 2010, 'Poly-b-hydroxybutyrate production by *Pseudomonas* sp. RSZ 1 under aerobic and semiaerobic condition', *Indian Journal of Express Biology*. vol. 48, pp. 942-947.
22. Yunus, AMM, Parveez, GKA & Ho, CL 2008, 'Transgenic Plants Producing Polyhydroxyalkanoates', *Asia Pacific Journal of Molecular Biology and Biotechnology*. vol. 16, pp. 1-10.
23. Parshad, I, Garson, T, Patel, JJ & Wong MN, 2001, 'The regulation of PHB metabolism in *Azotobacter beijerinckii*', *Applied Environmental Microbiology*. vol.125, pp. 75-78.
24. Kumbhakar, S, Singh, PK & Vidyarthi, AS 2012, 'Screening of root nodule bacteria for the production of polyhydroxyalkanoate (PHA) and the study of parameters influencing the PHA accumulation', *African Journal of Biotechnology*. vol. 11, no.31, pp.7934-7946.

25. Tripathi, AD & Srivastava, SK 2011, 'Kinetic study of biopolymer (PHB) synthesis in *Alcaligenes* sp. in submerged fermentation process using TEM', *Journal of Polymer Science and Environment*. vol. 19, pp. 732–738.
26. Tian, JA, Sinsky, J & Stubbe, J 2005, 'Kinetic studies of polyhydroxybutyrate granule formation in *Wautersiaeutropha*H16 by transmission electron microscopy', *Journal of Bacteriology*. vol. 187, pp. 3814-24.
27. Rohini, D, Phadnis, S & Rawal, SK 2006, 'Synthesis and characterization of polyhydroxybutyrate from *Bacillus thuringiensis* R1', *Indian journal of Biotechnology*. vol 5, pp. 276-283.
28. Sheu, DS, Wang, YT & Lee, CY 2000, 'Rapid detection of polyhydroxyalkanoate accumulating bacteria isolated from the environment by colony PCR.Microbiology', vol. 146, pp. 2019-2025.
29. Yuksekdag, ZN, Aslim, B, Beyadlti, X & Mercan, N 2004, 'Effect of carbon and nitrogen sources and incubation time on polyhydroxybutyrate (PHB) synthesis by *Bacillus subtilis*25 and *Bacillus megaterium*12', *African Journal of Biotechnology*. vol. 3, no. 1, pp. 63-66.
30. Aysel, U, Sahin, N & Beyatli, Y 2002, 'Accumulation of Poly- $\beta$ -Hydroxybutyrate in *Streptomyces* Species during Growth with Different Nitrogen Sources', *Turkey Journal of Biology*. vol. 26, pp. 171-174.
31. Divyashree, M, Rastogi, N & Shamala, T 2009, 'A simple kinetic model for growth and biosynthesis of polyhydroxyalkanoate in *Bacillus flexus*', *New Biotechnology*. vol. 26, pp. 92-98.
32. Preeti Yadav, Harsh Yadav, Veena Gowri Shah, Gaurav Shah, and Gaurav Dhaka Biomedical Biopolymers, their Origin and Evolution in Biomedical Sciences: A Systematic Review | *Clin Diagn Res*. 2015 vol 9 pp.21-25.
33. Naranjo, JM, Posada, JA, Higueta, JC & Cardona, CA 2013, 'Valorisation of glycerol through the production of biopolymers: The PHB case using *Bacillus megaterium*', *Bioresource Technology*. vol. 133, pp.38-44.
34. Saha, SP, Patra, A & Paul, AK 2005, 'Production of poly (3-hydroxybutyrate-co-3- hydroxyvalerate) by *Azotobacterchroococcum*MAL-201', In: 44th Annual Conference of Association of Microbiologists of India. pp. 80.
35. Omar, S, Rayes, A, Eqaab, A, Vo, I & Steinbuchel, A 2001, 'Optimization of cell growth and P3HB accumulation on date syrup by a *Bacillus megaterium* strain', *Biotechnology letters*. vol. 23, pp. 1119-1123.
36. Khardenavis, A, Sureshkumar & Chakrabarti, J 2003, 'Biodegradable plastics production from industrial waste water by activated sludge', In: 44th Annual Conference of Association of Microbiologists of India. pp. 107.
37. Bonatto, D, Matias, F, Lisboa, MP, Bogdawa, HM & Henriques, JAP 2004, 'Production of short side chain-poly[hydroxyalkanoate] by a newly isolated *Ralstoniapicketti* strain', *World Journal of Microbiology and Biotechnology*. vol. 20, pp. 395-403.
38. Jiang, Y, Song, X, Gong, L, Li, P, Dai, C & Shao, W 2008, 'High poly ( $\beta$ -hydroxybutyrate) production by *Pseudomonas fluorescens* A2a5 from inexpensive substrates', *Enzyme and microbial technology*. vol.42, pp. 167-172.
39. Kim, BS 2000, 'Production of poly (3-hydroxybutyrate) from inexpensive substrates', *Enzyme and Microbial Technology*. vol. 27, pp. 774-777.
40. Liu, JZ, Weng, LP, Zhang, QL, Xu, H & Ji, LN 2003. 'A mathematical model for gluconic acid fermentation by *Aspergillus niger*', *Biochemical Engineering*. vol. 14, pp. 137–141.
41. Silva, L, Taciro, M, Michelin, RM, Carter, J, Pradella, J & Gomez, J 2004, 'Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate', *Journal of industrial microbiology and biotechnology*. vol.31, pp.245-254.
42. Ahn, WS, Park, SJ & Lee SY 2000, 'Production of Poly (3-Hydroxybutyrate) by Fed-Batch Culture of Recombinant *Escherichia coli* with a Highly Concentrated Whey Solution', *Applied and environmental microbiology*. vol.66, pp.3624-3627.
43. Koller, M, Bona, R, Chiellini, E, Fernandez, EG, Horvat, P, Kutschera, C, Hesse, P & Brauneegg, G 2008, 'Polyhydroxyalkanoate production from whey by *Pseudomonas hydrogenovora*', *Bioresource Technology*. vol. 99, pp. 4854-4863.
44. Nath, A, Dixit, M, Bandiya, A, Chavda, S & Desai, AJ 2008, 'Enhanced PHB production and scale up studies using cheese whey in fed batch culture of *Methylobacterium* sp.ZP24', *Bioresource Technology*. vol. 99, pp. 5749-5755.
45. Vijayendra, SVN, Rastogi, NK, Shamala, TR, Kumar, PKA, Kshama, L & Joshi, GJ 2007, 'Optimization of polyhydroxybutyrate production by *Bacillus* sp. CFR 256 with corn steep liquor as a nitrogen source', *Indian Journal of Microbiology*. vol. 47, pp. 170-175.
46. Chaijamrus, S & Udpuay, N 2008, 'Production and Characterization of Polyhydroxybutyrate from Molasses and Corn Steep Liquor produced by *Bacillus megaterium* ATCC 6748', *Agricultural Engineering International Manuscript*, vol.7, no. 30, pp. 10.
47. Daneshi, A, Younesi, H, Ghasempouri, M & Sharifzadeh, M 2010, 'Production of poly-3-hydroxybutyrate by *Cupriavidus necator* from corn syrup: statistical modeling and optimization of biomass yield and volumetric productivity', *Journal of Chemical Technology and Biotechnology*. 85(11):1528-1539.
48. Madhumathi, R, Muthukumar, K, & Velan, M, (2016) "Optimization of Polyhydroxybutyrate production by *Bacillus safensis* EBT1", *Clean-soil, Air, Water*. 44(8):1066-1074
49. Kek, YK, Chang, CW, Amirul, AA & Sudesh, K 2010, 'Heterologous expression of *Cupriavidus* sp. USMAA2-4 PHA synthase gene in PHB-4 mutant for the production of poly (3-hydroxybutyrate) and its copolymers', *World Journal of Microbiology and Biotechnology*. 26(9):1595-1603.

50. Kahar, P, Tsuge, T, Taguchi, K & Doi, Y, 2004, 'High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain', *Polymer degradation and stability*. 83:79-86.
51. Fernandez, D, Rodriguez, E, Bassas, M, Vinas, M, Solanas, AM, Llorens, J, Marques AM & Manresa, A 2005, 'Agro-industrial oily wastes as substrates for PHA production by the new strain *Pseudomonas aeruginosa* NCIB 40045: Effect of culture conditions', *Biochemical Engineering Journal*. 26:159-167.
52. Cavalheiro, JMBT, Almeida, MCMD, Grandfils, C, & DaFonseca, MMR 2009, 'Poly (3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol', *Process Biochemistry*. 44:509-515.
53. Bengtsson, S, Werker, A, Christensson, M & Welander, T 2008, 'Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater', *Bioresource Technology*. 99:509-516.
54. Choi, KS, Ryu, HW, Park, CH & Goodrich, PR 1997, 'Poly (hydroxybutyrate-co-hydroxyvalerate) from swine waste liquor by *Azotobacter vinelandii* UWD', *Biotechnology Letters*. 19: 7-10.
55. Goff, M, Ward, PG & Connor, KE 2007, 'Improvement of the conversion of polystyrene to polyhydroxyalkanoate through the manipulation of the microbial aspect of the process: a nitrogen feeding strategy for bacterial cells in a stirred tank reactor', *Journal of Biotechnology*. 132(3):283-286.
56. Wang, L, Wang, ZH, Shen, CY, You, ML, Xiao, JF & Chen, GQ 2010, 'Differentiation of human bone marrow mesenchymal stem cells grown in terpolyesters of 3-hydroxyalkanoates scaffolds into nerve cells', *Biomaterials*. 31(7):1691-1698.
57. Thirumala, M, Reddy, SV & Mahmood, SK 2010. Production and characterization of PHB from two novel strains of *Bacillus* spp. isolated from soil and activated sludge', *Journal of Industrial Microbiology and Biotechnology*. 37:271-278.
58. Pandian, S, Deepak, V, Kalishwaralal, K, Rameshkumar, N, Muniyandi, J & Gurunathan, SS 2010. Optimization and fed-batch production of PHB utilizing dairy waste and sea water as nutrient sources by *Bacillus megaterium* SRKP-3, *Bioresource Technology*. 101:705-711.
59. Ribera, RG, Monteoliva, SM & Ramos, CA 2001, 'Production of polyhydroxyalkanoates by *Pseudomonas putida* KT2442 harboring pSK2665 in wastewater from olive oil mills (alpechin)', *Electronic Journal of Biotechnology*. 4:116-119.
60. Pozo, C, Martinez, TMV, Rodelas, B & Gonzalez LJ 2002. Effects of culture conditions on the production of polyhydroxyalkanoates by *Azotobacter chroococcum* H23 in media containing a high concentration of alpechin (wastewater from olive oil mills) as primary carbon source, *Journal of Biotechnology*. 97: 125-131.
61. Sathiyarayanan, G, Kiran, GS, Selvin, J & Saibaba, G 2013. Optimization of polyhydroxybutyrate production by marine *Bacillus megaterium* MSBN04 under solid state culture, *International Journal of Biological Macromolecules*. 60:253 - 261.
62. Law, K.H, Cheng, Y.C, Leung, Y.C, Lo, W.H, Chua, H & Yu, H.F 2003. Construction of recombinant *Bacillus subtilis* strains for polyhydroxyalkanoates synthesis, *Biochemical Engineering Journal*. 16:203-208.
63. Yezza, A, Halasz, A, Levadoux, W & Hawari, J 2007. 'Production of poly-hydroxybutyrate (PHB) by *Alcaligenes latus* from maple sap', *Applied Microbiology and Biotechnology*. 77:269-274.
64. Zafar, M, Kumar, S, Kumar, S & Dhiman, AK 2012. Optimization of polyhydroxybutyrate (PHB) production by *Azohydromonas lata* MTCC 2311 by using genetic algorithm based on artificial neural network and response surface methodology, *Biocatalysis and Agricultural Biotechnology*. 1:70-79.
65. Rajendran, R, Mekala, M & Suganya, K 2013. PHB production by *Bacillus* species using the cheap substrate groundnut oil cake, *International Journal of Pharma and Bio Sciences*. L(1): 1006 - 1015.
66. Gumel, AM, Annuar, M.S.M & Heidelberg, T 2012. Biosynthesis and Characterization of Polyhydroxyalkanoates Copolymers Produced by *Pseudomonas putida* Bet001 isolated from Palm Oil Mill Effluent, *PLoS ONE*. 7(9):e45214.