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# Polyhydroxybutyrate Production by various Substrates: Optimization and Application

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**ABSTRACT: Synthetic plastics because of their non-biodegradability have made huge waste accumulation resulting in hazardous environmental issues that must be controlled soon. There is a crucial need for the substitution of these plastics with biodegradable plastic. Polyhydroxybutyrate (PHB) is one of the emerging biopolymers which can be a perfect and eco-friendly substitution for synthetic plastics. Due to its unique properties, it can be a better alternative to synthetic plastic. Since plastics accumulate millions of tons annually in the environment and cause life-threatening problems, therefore the production of PHB using low-priced substrates is important. The present research has shown that carbon sources were found to be a pivotal factor in the production of PHB. The present article focuses on the production of PHB through various microbes and economical feedstocks, its optimization, and the applications of PHB in different areas. Various challenges have been faced in PHB production such as high production cost, low thermal stability, physical aging problem, low yield, difficulties in extraction, etc. Such serious problems have been tackled wisely. Cheap substrates and microbes are used for PHB production.**

**Keywords:** Bio-degradable plastic, Poly-3-hydroxy-butyrate, Physicochemical technique, Alkali pre-treatment, Bio-composites.

## INTRODUCTION

The use of large quantities of non-degradable traditional plastics, production, and disposal, increases the amount of municipal and industrial waste which is among the major global issues nowadays. Biodegradable plastics from biopolymers such as Poly-β hydroxybutyrate (PHB) produced by microorganisms are potential substitutes for these non-degradable petroleum-based plastics (Amadu *et al.,* 2021). It is an emerging biopolymer since it has lots of potential and applications because of its eco-friendly nature, non-toxicity, biocompatibility, and various unique properties, that make it better than other polymers (Sharma, 2019; Livshits *et al.,* 2009).

Numerous kinds of research are ongoing on PHB in the present time, so that scientists and manufacturers could understand its different properties and various new applications which will ultimately benefit the environment and humankind greatly in the upcoming future. PHB is an environmentally free biopolymer that can be produced from various renewable resources and has a wide range of applications in various fields. Since there is numerous importance of PHB, therefore its manufacturing must be vast and cost-effective so that it could be easily available and will fully replace traditional plastic in the upcoming future. They are produced from various microbes, feedstocks, and wastes such as agricultural waste materials (Getachew and Woldesenbet 2016), municipal waste (Yuan *et al.,* 2015), etc.

**History of PHB.** Polyhydroxybutyrate (PHB) was discovered by Maurice Lemoigne in 1926 (Lenz and Marchessault 2005). He discovered that Poly 3-hydroxybutyrate is produced by joining β-Hydroxybutyrate bonds through ester bonds (Yadav and Prabha 2017). Polyhydroxybutyrate, a family of Polyhydroxyalkanoates (PHA) is the first discovered polymer among the PHA family which is derived from the class of polyesters having various unique properties which have various useful applications in different sectors. A wide range of various bacterial stains were examined which could accumulate PHB-related organisms and laboratory stocks and it was observed that it could provide a reserve of carbon and energy and accumulates as intracellular granules when surplus carbon is available, but at that time the growth was restricted due to some factors such as the availability of essential nutrients, etc (Haywood *et al*., 1989).

**Properties of PHB.** Polyhydroxybutyrate (PHB) has various unique properties, because of which it can be widely used in different fields as biodegradable plastics, in the pharmacological, medical, and agricultural fields, in food packaging, etc. Poly(3-hydroxybutyrate) (PHB) and its copolymers with 3-hydroxyvalerate (HV) having a chemical structure of $[OCH(CH_3)CH_2(CO)]n$ and $[OCH(CH_3)CH_2(CO)]x$ $[OCH(C_2H_5)CH_2(CO)]y$ which are synthesized by bacteria. PHB has attracted the interest of lots of researchers and scientists because of its unique properties like biocompatibility, biodegradability, less toxicity, UV resistance, etc. They have relatively high melting temperatures (Tm), up to 170°C for the homopolymer (Gunaratne and Shanks 2005). PHB has various unique properties such as brittleness, good barrier properties, low thermal stability, high degree of crystallinity, etc. Some of those properties are similar to that of Polypropylene (PP) and Polythene (PE) also. Due to its special properties, it can replace that traditional polyethylene. The glass transition temperature (Tg) of PHB is around 5°C and the crystalline melting temperature (Tm) is almost 180°C similar to the properties found in polypropylene (Rodriguez *et al.,* 2021). Due to all these unique properties of the PHB, they can be easily used as an alternative to plastic with slight modification.

## POLYHYDROXYBUTYRATE PRODUCTION USING VARIOUS SUBSTRATES AND THEIR STRAINS

The main hassle in the manufacturing of PHB using physicochemical technique is themanufacturing cost as compared to the petrochemical obtained plastic production.

Optimizing various inexpensive substrates to overcome manufacturing costs in large-scale commercial production is one of the well-known trends in the industry today.

**Polyhydroxybutyrate production using sugarcane molasses as substrate by *Bacillus cereus* 2156.** The waste from the sugar mills can be used as a substrate for the production of polyhydroxybutyrate. In this study, Bacillus cereus 2156 (NCIM 2156) obtained from National Chemical Laboratory Pune was used.

Sudan black staining method was used for screening of pure culture of Bacillus cereus for the production of Polyhydroxybutyrate (PHB) which was suggested by the researchers (Suryawanshi *et al.,* 2020). This process includes a collection of a small amount of sugarcane molasses and then air drying it; followed by keeping it in a zipper airbag and storing it. Before this procedure, the sampled molasses needed to be autoclaved. Furthermore, this sample is used a carbon source.

The response surface methodology is best used to optimize the production of PHB. To optimize some physiochemical parameters to escalate PHB synthesis Plackett- Burman screening design (Plackett *et al*., 1946) was brought up into action. A few limits which were enlisted for affecting the production of PHB were AX-carbon source (molasses), BX- nitrogen source (urea), CX-Mg source, CX-Mg source ($MgSO_4.7H_2O$), and DX-pH.

To conclude the particular and liberated effect of the variable on the response and various factor relations a Box and Behnken (1960) design was used. Quantitative analysis of polyhydroxybutyrate was conducted by using UV-Vis spectrophotometer at 235 nm by this mechanism percentage PHB production was determined and crotonic acid was taken as a standard (Law and Slepecky 1961). To verify the optimum manufacturing of PHB, medium and culture conditions were optimized. Extracted fabricated form of PHB granules was dissolved in chloroform and then layered on a NaCl crystal (Kelly and Downey 2005).

The response surface methodology is best used to optimize the production of PHB. To optimize some physiochemical parameters to escalate PHB synthesis Plackett-Burman screening design (Plackett *et al.,* 1946) was brought up into action. The results show that total PHB productivity is between 50% and 60% of total biomass. These results were significantly better than in previous studies (Gomaa, 2014). Manufacturing Polyhydroxybutyrate by consuming cheap boards makes the process economically viable. Ultimately, this optimized media enhances the ability to scale pilot and industrial scale PHB production for a variety of applications, demonstrating that environmentally friendly PHB polymers can be produced from environmentally friendly waste.

**Canned pineapple as a substrate by Cupriavidus necator strain A-04.** Amongst multiple green products that are currently available, Lignocellulosic has become the best alternative for the production of several biofuels and reagents including PHAs. Cellulose resources include Agriculture and Forestry Residues, and urban and industrial waste presented as low-value renewable resources representing viable options for producing high-value products (Kunaver *et al.,* 2016).

Pineapple (*Ananas comosus* L. Merr.) is one of the most profitable fruit and it is produced worldwide. Estimates about 24.8 million tons of pineapple are produced annually all over the world.

During the processing of canned food high amount of agricultural industrial waste and pineapple, waste is produced. The product contains 44.36% peel and 15% core for all raw materials (Sukruansuwan and Napathorn 2018).

A large amount of biochemical oxygen demand is required for the digestion of pineapple and it also produces methane if proper waste management practices are not implemented. It can cause greenhouse gas emissions. Agricultural industrial residues from the canning industry can be used for the production of biodegradable PHBs. Canned pineapple industrial waste as a raw material for lignocellulosic feedstocks is feasible for manufacturing Polyhydroxybutyrate. C. necator strain A04 was able to grow on a variety of sugars, tolerate levulinic acid and 5-hydroxymethylfurfural and did not require a detoxification step before converting the cellulose hydrolysate to PHB. Pineapple cores are classified into -20/+40 mesh particles and hydrolyzed with 1.5% (v/v) $H_2SO_4$ to produce the highest concentration of fermentable sugar equal to 0.81 g/g dry pineapple kernels, +20 mesh. Generated a pineapple kernel. Hydrolysis with particle size and 1.5% (v/v) $H_3PO_4$ gave the highest concentration of PHB substrate (57.2 ± 1.0 g/L). Total PHB production from core hydrolysate was 35.6 ± 0.1% (w/w) PHB content and 5.88 ± 0.25 g/cell dry weight. We investigated the use of pineapple by-products (skin and core) as a medium for crude aqueous extract (CAE) produced a substrate, and 0.160 g/(Lh) productivity (Du *et al*., 2010). In addition to acid hydrolysis, CAE has been found as a good source of carbon, providing a new way to inexpensively produce PHB from realistic lignocellulosic biomass raw materials.

**Poly (3-hydroxybutyrate) production using cheese whey as a substrate by *Bacillus megaterium* NCIM 5472.** A large percentage of milk products are cheese whey which is part of nutritional vitamins for human beings. Since maximum whey constituent molecules are of small molecular weight and soluble which includes lactose, minerals, bovine serum albumin, β-lactoglobulin, etc, they can rapidly lower oxygen levels in herbal water bodies (Koller *et al*., 2008; Yellore and Desai 1998). When cheese whey is passed through an ultrafiltration membrane to distill whey protein we obtain whey permeate as a by-product (Baldasso *et al.,* 2011).

This permeate element may be applied as a reasonably-priced carbon supply for the manufacturing of PHAs because of the better share of lactose found in permeate. *B. megaterium* is a rod-shaped, gram-positive aerobic spore-forming bacterium that is endemic in a variety of habitats. Recently, its popularity in the field of biotechnology has increased due to its ability and capacity to produce recombinant proteins (Bunk *et al.,* 2010). *B. megaterium* NCIM5472 was bought from NCIM, Pune, India; furthermore Sudan black B and Nile blue A staining methods were used to examine the presence of intracellular PHB granules (Chaudhry *et al.,* 2011). This permeate can be used effectively, and after optimizing various process parameters, it is observed that the bacteria can accumulate 75.5% PHB of dry weight and produce a high PHB yield of 8.29 g/L (Wang *et al.,* 2013). This by-product can make a big difference in the biopolymer production industry and can significantly reduce investment in carbon sources.

Further modifications can be implied to produce polymers with different thermal and mechanical properties to develop a fully sustainable PHB production process using cheese whey.

**Polyhydroxybutyrate production using cow dung as a cheap carbon source by Bacillus Pumilus H9.** Cow dung substrates are an affordable and readily available carbon source for the production of PHB and have been effectively used as a vehicle for bacterial growth (Oranusi *et al.,* 2014). The chemical composition of the cow dung includes 1.6% N, 0.70% P, 0.53% K, 0.91% Mg, 2.71%, 0.50% Na, 56.8% organic material, and C: N of 7.9 (Onwudike*,* 2010). Proper media formulations for PHB's maximal production are important from the industrial perspective as intermediate parts have a major impact on product yields. Ideal substrates should be available throughout the year (Pandey *et al.,* 2000).

Various bacterial strains that effectively accumulate PHB were isolated from soil samples in the municipal waste area of Hailakandi in Assam. The screening was performed by the Sudan Black B staining method, and positive isolates showed blue-black stained cytosol in bacterial cytosol. An effective PHB producer was selected based on the dry weight of the extracted PHB. An effective PHB producer was selected based on the dry weight of the extricated PHB. The H9 strain has the highest PHB content of any other bacterium and was therefore selected for further study.

In consideration of simple availability, PHB was produced from *Bacillus pumilus* H9 as a low-cost substrate (Weisburg *et al*., 1991). Gram-positive bacteria (designated H9 strain) found to be potential polyhydroxybutyrate (biodegradable polymer) producers were isolated from the soil

samples in stress-prone environments (urban waste areas). The bacterium *Bacillus pumilus* H9 went through a four-element central composite rotation design to optimize the medium and find the interactive effects of the four variables. Concentrations and pH of cow dung, sucrose, and peptone in PHB production. Using response surface methodology, multiple regression analysis yielded a quadratic polynomial with a pH 7 optimized medium yielding a 2.47 g/L of PHB dry weight yield. Here we report on cow dung as an inexpensive carbon source for producing PHB. Furthermore, it was confirmed that the phbA, phbB, and phbC genes were amplified by the polymerase chain reaction, allowing bacteria to produce polyhydroxybutyrate (Omidvar *et al.,* 2008).

**Poly β-Hydroxybutyrate production using sugar industry waste water by *Bacillus subtilis* NG220.** As we know, the amount of wastewater produced during sugar production is constantly expanding. The wastewater includes floor-washing wastewater and condensate, sugar cane juice, syrup, molasses, and more. Wastewater from sugar mills has high biochemical oxygen demand (BOD), and when discharged untreated, increased BOD affects aquatic ecosystems and ultimately human health. The sugar industry has to spend a lot of money on wastewater treatment to meet legal standards. Therefore, the two distinct problems of pollution by synthetic plastics and the production of organic waste from the sugar industry can be addressed independently by producing bioplastics that use organic waste as a nutrient source. However, this carbohydrate waste is deficient in nitrogen concerning certain mineral nutrients, especially the amount of oxidizable carbon present, and may require nutrient supplementation. Here, after adjusting the nutrients, wastewater from the sugar industry was used for the production of PHB using the isolate *Bacillus subtilis* NG220.

*Bacillus subtilis* NG220 was isolated in carbon-rich nutritional agar medium [(w/v) glucose 1%, 0.3% beef extract, 0.5% peptone, 0.8% of chloride, agar 1.5%] from the sugarcane Field sector. The presence of sugar cane intracellular PHB granules from soil samples was confirmed by staining with Sudan Black B and Nile Blue A (Bänziger *et al*., 2001).

Cultures were maintained on nutritional agar, bovine and then stored at 4°C. The isolate produced 5.297 g/L of poly-β-hydroxybutyrate (Kim *et al*., 2006). In biodegradable studies, polymer PHB films (prepared by conventional solvent casting techniques) are used in compost containing 25% (w/w) water after 30 days, with various natural sources such as soil, compost, and industrial sludge (Sangkharak *et al.,* 2008). Resisted decomposition in the habitat. From this, we can see the benefits of converting waste into various outcomes, as well as insights into PHB and waste management.

**Poly (3-hydroxybutyrate) production using banana (Musa sp.) fronds extract by Cupriavidus necator H16.** Banana trees are an important renewable resource. Banana leaf extract (BFE) contains a significant amount of renewable sugar. 16.6 g/L glucose and fructose make up 55% of all fermented sugars (Chai *et al.,* 2018). BFE was chosen as the raw material for making PHA. Therefore, in this fermentation study, banana tree waste is advertised as a suitable biomass material as a carbon source.

In this study, we used a wild strain of Cupriavidus necator H16 (ATCC 17699), which can consume fructose as a carbon source. This strain was stored in 40% (v/v) glycerol at - 80°C. The sucrase enzyme baker's yeast (*Saccharomyces cerevisiae*) was obtained from Sigma

Aldrich. Furthermore, the formation of PHB was confirmed by Nile red staining of PHB granules and visualization with a fluorescence microscope.

The effects of various BFE concentrations for PHB preparations were incubated for 72 hours by fermenting BFE at 30°C for 10 to 50% (v/v). Fermentation with 40% (v/v) BFE resulted in the highest PHB content in biomass (32.1%) and PHB concentration (0.9 g/L). Furthermore, the production of PHB was also examined by comparing fermentation with 40% (v/v) RAW and SUCRASE enzyme BFE. The fructose content in the enzyme pre-treatment BFE after enzyme hydrolysis rose to 14.6 g/L. 40% (v/v) Pre-treated fermentation using BFE is a higher PHB of biomass (37.4%) and PHB concentration (1.3 g/L) as compared to the original BFE of 40% (V/V) (Zahari *et al*., 2014). The results show that 40% (v/v) of enzyme-pre-treated BFE can be used as an alternative renewable and sustainable carbon source for the manufacturing of Polyhydroxybutyrate.

**Table 1: Methods used for screening of various strains of pure cultures.**

| Sr. No. | Strains | Methods |
|---|---|---|
| 1. | *Bacillus cereus* 2156 | Sudan Black staining (Suryawanshi *et al.*, 2020) |
| 2. | *Cupriavidus necator* strain A-04 | Nile red staining |
| 3. | *Bacillus megaterium* NCIM5472 | Sudan Black B and Nile blue A (Chaudhry *et al.*, 2011) |
| 4. | *Bacillus Pumilus* H9 | Sudan Black B |
| 5. | *Bacillus subtilis* NG220 | Sudan Black B and Nile blue A (Bänziger *et al.*, 2001) |
| 6. | *Cupriavidus necator* H16 | Nile red staining |

## OPTIMIZATION STRATEGIES FOR PHB PRODUCTION

### Selection of an efficient biomass pre-treatment method for fruit waste as a Substrate

Various useful technologies have been developed and are used for the pre-treatment of lignocellulosic biomass. These methods aim to disrupt the complex lignin, cellulose, and hemicellulose network in the cell wall to enhance enzyme accessibility for further hydrolysis (Sirohi *et al*., 2021). Lignocellulosic biomass contains some remnants of agricultural wastes or different biomasses. The industrial processing of different fruits for juice, pulp, etc. generates large quantities of residues like peel, core, stem, and crown which are generally utilized as manure or animal feed. These fruit wastes are rich in fermentable sugars and hence can be exploited for the production of valuable products including biofuel like Polyhydroxybutyrate. Here are Different pre-treatment strategies which can increase PHB production:

**Pre-treatment by Alkali.** Pre-treatment with alkali is a cost-effective, energy-intensive, and straightforward approach for selectively removing lignin from lignocellulosic biomass without significantly affecting the composition of reducing sugars and carbohydrates. It is used for various feedstocks including agricultural residues and forages and is relatively cheaper than other pre-treatment methods (Chen *et al*., 2007; Xu *et al.,* 2010). Alkaline pre-treatment allows for better enzyme penetration and hydrolysis as a result of the increased porosity and surface area in the cell wall structure (Kim *et al.,* 2016). Alkaline conditions disrupt the alkyl-aryl linkages present in lignin, de-crystallization of cellulose, and de-polymerization of the carbohydrates present in the biomass structure (Mosier *et al.,* 2005; Park and Kim 2012).

**Pre-treatment by Microwave.** The use of microwaves for pre-treatment of lignocellulosic biomass shows the blessings of lower activation energy and shorter response time (Kang *et al*., 2019). The direct touch among the electromagnetic discipline of microwaves and biomass results in extra targeted and rapid heating of a huge quantity of the product compared to conventional heating methods (De La Hoz *et al.,* 2004). Microwave irradiation can go into the complicated and inflexible lignocellulosic structures, disrupt them and disclose the lignocellulosic biomass for higher enzymatic hydrolysis (Chen *et al.,* 2011). For higher ease of running with microwaves as a pre-remedy technology, vessels, and reactors were evolved to make effective biomass loading and microwave response (Li *et al*., 2016; Ooshima *et al.,* 1984).

**Pre-treatment by Steam explosion.** This is a highly efficient thermos-physical-chemical technique that can alter the lignocellulosic structure's dissociation of hemicellulose and lignin. This process combines steam decomposition and an explosive decompression step to mechanically destroy the lignocellulosic biomass structure (Maniet *et al*., 2017). In this process, the biomass is treated with a controlled residence time under high-pressure saturated vapor, then the pressure is rapidly released to cause an explosive depressurization, destroying the cell wall structure and solubilizing the hemicellulose and lignin components (the hemicellulose and lignin components are solubilized (Bonfiglio *et al.,* 2019).

**Effect of Temperature on PHB Production.** Incubation temperature affects PHB production by altering the enzymes involved in the production process (Getachew and Woldesenbet 2016). Use of palm honey syrup in the manufacture of PHB from various marine bacteria has been documented. Throughout the fermentation process, temperature ranges of 25, 30, 35, 40, and 45°C were investigated. After incubation, the highest biomass was achieved at 35 degrees Celsius. In addition, temperature changes have been observed to have a strong effect on PHB production and bacterial growth. PHB production was 4.38 g/L and biomass synthesis was 9.23 g/L, corresponding to 47.45 percent total PHB content and 43.8 percent PHB yield, respectively. *Bacillus dentensis* strains were injected and cultured for 36 hours at various temperatures ranging from 25 to 37°C, with the highest yields and accumulations of PHB occurring at 28°C. Strains could not grow below 28°C and above 28°C (Penkhrue *et al.,* 2020).

*Bacillus subtilis* was also used in the manufacture of PHBs utilizing fruit peel infusions to increase biopolymer synthesis. Temperature has been highlighted as a key element that needs to be optimized among all the characteristics related with the medium. The incubation temperature was varied from 25 to 50°C in this study, and *Bacillus subtilis* produced up to 3.35 g/L PHB at 35°C, the highest of the temperatures tested. Temperatures above 40°C have been found to be fatal to microbial growth and therefore ineffective in PHB production (Santhi and Balakumaran 2018). When a Bacillus subtilis strain isolated from the wastewater sample was treated with a fruit peel extract as a carbon source, it produced more PHB. During the production process, certain physicochemical parameters were evaluated in order to boost the action of bacterial strains that manufacture PHB on a bigger scale. The temperature ranges tested were 27, 32, 37, 42, and 45°C, out of which, the maximum amount of PHB (336 µg/ml) obtained at a temperature of 37°C (Irsath *et al.,* 2015).

**Effect of pH on PHB Production.** Like temperature, pH is another important parameter for the fabrication of biopolymers. The lodgment of PHB in bacterial cells has been shown to be affected by changes in pH levels. Understanding the increased synthesis of PHB requires an understanding of the PHB polymerase enzyme's function. PHB creation has been linked to the concentration of hydrogen ions in the medium, which can interfere with the metabolic pathways of the microbes that produce it. Practical were carried out by adjusting the pH of the fermentation medium from 6.5 to 9.0 to evaluate the influence of pH. At pH 7.5, the greatest PHB concentration of 4.41 g/L and bacterial biomass of

9.26 g/L were achieved. B. drentensis used pineapple extract juice to raise the pH for optimal PHB output. The concentration of PHB generated was significantly different in different pH ranges after adjusting the pH of the fermentation medium from 5 to 10 and incubated for 72 hours. Maximum PHB yields (3.7 g/L) were observed after 36 hours of incubation at pH 6, followed by pH 6.5 and 7.0 (Penkhrue *et al.*, 2020). Another study by Santhi and Balakumaran (2018) optimized the pH of the medium during PHB production from the pericarp extract. At pH 7, a maximum yield of 3.21 g/L of PHB was obtained. Changing the pH of the medium from 7 to 8 and 8 to 9 significantly altered PHB production. In addition, an optimization strategy by changing the pH of the medium maximized PHB production by *Bacillus subtilis* at pH 7.5, resulting in a yield of 362 µg/mL (Irsath *et al.,* 2015).

**Effect of Carbon Source Present in Media on PHB Production.** PHBs made from rice bran and glucose-based carbon sources were recently distinguished, with results showing that PHBs made from rice bran carbon sources were chemically similar to commercial PHB materials, with higher thermal stability and lower melting point than glucose carbon sources throughout PHB material production (Hassan *et al.,* 2019). Through various experiments, it was seen how organic carbon sources affected the production of autotrophic metabolic enzymes. It was found that the organic carbon source influenced the PHB materials' attributes, with changes in thermal properties and crystallinity stated (Garcia Gonzalez *et al*., 2015). Research also compared autotrophic and heterotrophic production (using $CO_2$ as a carbon source and $H_2$ as an energy source) and typical heterotrophic growth (using organic substances as a carbon source and source of energy). There was a difference between the two groups, with autotrophic production having greater $T_m$, $T_g$, higher degree of crystallinity, and lower $T_d$ values than PHB manufactured by the heterotrophic procedure (Garcia Gonazalez *et al*., 2015). These findings show that further improving PHB yield features and tailoring their thermo-mechanical performance to compete with petroleum-based Polythene and Polypropylene polymers at competitive pricing is a strong possibility.

## 8 EFFECT OF MICROBIAL LOAD ON PHB PRODUCTION

PHB production was noted to be affected by the density of inoculum in production media. To reform the inoculum ratio in the medium, the production of PHA by B. dentrensis was observed by some researchers (Penkhrue *et al*., 2020). It was observed that when different concentrations of inoculants, namely 4, 6, 8, 10, and 20% (v/v) were poured into the production flask, the 2% inoculum size produced less biomass and PHB. The PHB content got an increment of 3.1 to 4.1 g/L when the inoculum size was expanded from 4% to 10%.

## RECENT DEVELOPMENT ON POLYHYDROXYBUTYRATE (PHB)

In these recent years, researchers and scientists have done different kinds of developments and modifications on PHB for its better production and use. Researchers are developing and advancing PHB for its future benefits. In this whole process of development, these researchers and scientists have faced numerous challenges like limited PHB production, high cost of processes etc, and finding solutions to tackle it.

**PHB development through Cyanobacteria.** Cyanobacteria are photosynthetic bacteria having high protein content, essential vitamins, and minerals. Due to their unique properties, they are used as food supplements and biopolymers. Cyanobacteria can generate PHB intercellularly and store it as a source of energy using sunlight and CO2. Cyanobacterial PHB is having best qualities as compared with any other prokaryotic PHB. Cyanobacteria can grow in wastewater so they can utilize excess nitrogen and phosphate for growth and accumulation of PHB from wastewater hence by reusing the wastewater. Depending on the cultivation condition, Cyanobacteria can easily duplicate their biomass in 24 hours and are also capable of producing their biopolymers. Several carbon sources are also been studied for the production of Cyanobacterial PHB, including $NaHCO_3$, PHB extraction residue, $CO_2$, glucose, and wastewater (Costa *et al*., 2018).

During the downstream processing of PHB recovery in extraction, it requires repetitive washing of pellets. Cyanobacteria contain many pigments that were having various negative effects on the properties of PHB. Therefore, pigment removal from the Cyanobacteria is important before PHB recovery. Here methanol could be used for those pigment removal. Methanol could be used with chloroform before PHB extraction (Yashavanth *et al*., 2021).

In general, PHB is produced using non-genetically engineered bacterial strains. Few transgenic strains have shown a good amount of mass-scale PHB production. In fact, the growth in fermentation approaches also exhibits production improvement (Jangra *et al.,* 2018).

**Development in the PHB production by *Cupriavidus necator* using $CO_2$.** The high cost of PHB production is a major problem because of the substrate used and the down-streaming process during its production for commercial use. Therefore, scientists have developed a cost-effective way of PHB production through carbon sources. PHB is produced by *Cupriavidus necator* by using $CO_2$ as a feedstock. Among low-cost feedstock, $CO_2$ is one of the best among them because of its availability, low-cost and being renewable feedstock. $CO_2$ is abundant in the environment and since it's a renewable feedstock, it could be easily used for the production of PHB. Some bacterial strains are producing PHB by utilizing $CO_2$ as an energy source. *Cupriavidus necator*, a β-proteobacteria produces PHB from $CO_2$ (Lee *et al.,* 2021). There were some challenges as $CO_2$ metabolizing bacteria showed limited PHB production and also it is little bit costly than the production of synthetic plastics. Therefore to tackle this problem, researchers have developed different strategies for PHB production.

**Development of different biomass feedstock for PHB production.** Various biomass feedstocks play an important role as a suitable substrate for the production of PHB. PHBs are produced from different feedstock such as sucrose-based materials, starch-based materials, glycerol, formic acid, acetic acid, dairy industries wastes, etc. Starch-based materials- Starch is hydrolyzed into simple sugars by amylase and glucoamylase and PHB is produced through microbes like *Halomonas boliviensis, Escherichia coli,* etc. Sucrose-based materials- Molasses are the cheapest source of carbon and can be used for PHB production through *Bacillus megaterium* BA-019 strain using low-cost resources (Sirohi *et al.,* 2020). PHB could be produced through the sugar refinery waste products that are having potential for applications in food packaging. The utilization of sugar refinery waste products for PHB production makes it 50% cost effective and beneficial for industrial purposes (Tripathi *et al.,* 2019). Different feedstocks are in use as substrates for the production of PHB from different reactions.

**Table 2: Comparison of different feedstock for PHB production in terms of PHB yield and $CO_2$ fixation through its Stoichiometric reactions (Vlaeminck *et al.,* 2022).**

| Sr. No. | Substrateused | Stoichiometric reactions | PHB yield (gPHB/ gS) | $CO_2$ fixation (g $CO_2$/gS) |
|---|---|---|---|---|
| 1. | $CO_2/H_2$ | $4\ CO_2 + 33\ H_2 + 12\ O_2 \rightarrow C_4H_6O_2 + 30\ H_2O$ | 0.36 | 0.73 |
| 2. | Glycerol | $2\ C_3H_7O_3 + 2.5\ O_2 \rightarrow C_4H_6O_2 + 5H_2O + 2\ CO_2$ | 0.47 | 0.48 |
| 3. | Formic acid | $33\ CH_2O_2 + 12\ O_2 \rightarrow C_4H_6O_2 + 30H_2O + 29\ CO_2$ | 0.06 | 0.12 |
| 4. | Acetic acid | $3\ C_2H_4O_2 + 1.5\ O_2 \rightarrow C_4H_6O_2 + 9H_2O + 2\ CO_2$ | 0.48 | 0.98 |

**Development of PHB-blown films.** PHB-blown films are made up of fabricated acidic acid as an alternative to chloroform. It is prepared through a solvent casting process at a temperature ranging from 80°C - 160°C and it is used for the drug delivery process and in food packaging. Scientists have developed PHB-blown films for better food packaging. Before processing, pellets of PLA (Polyhydroxyalkanoates) and PHB (Polyhydroxybutyrate) were dried under a vacuum at 70°C temperature for 16 hours. They are put at different PLA/PHB concentrations (100/0, 80/20, 70/30, 60/40 by weight) were melt-blended in a Collin ZK25 co-rotating twin extruder, with a screw speed of 100 rpm and having varying temperature ranges. Then, each blend was dried under vacuum at 70°C for 16 hours before starting the process. The blown films were prepared in a single screw extruder and for each system, the process conditions were optimized in order to produce blown films with a constant thickness of 45 µm (Pietrosanto *et al*., 2021). PLA (Polylactic acid) is a biodegradable material used for food packaging. PLA also has some disadvantages like high brittleness, high oxygen or water permeability. On the other hand, PHB is having high crystallinity and good barrier properties. Therefore it was found that blends of PLA/PHB blown films would be a better alternative for food packaging rather than the normal PLA.

**Developments in PHB-based Biocomposites.** In recent years, some modifications have been done in the synthesis of the PHB-based biocomposites. Biocomposites are blends of different natural materials that are fabricated by combining natural fibers. PHB-based biocomposites play a crucial role in food packaging. Some modification has been done with biodegradable PHB-based composites to increase its productivity (Chen and Wang 2002) such as the incorporation of PHV with PHB resulting in the copolymer of PHBV providing high ductility and strength, grafting of PHB and cellulose giving stable compound compared to ungrafted cellulose and PHB, Hydroxyperoxide (HA) mediated PHB composites have been developed for making dental implants (Szcześ *et al*., 2017), PHB-based starch blended biocomposites have been developed for promoting improved thermal and mechanical properties of PHB, making it fully biodegradable composites (Zhang and Thomas 2010).

**Toughening of PHB.** Though PHB has numerous desirable properties and has lots of applications in different fields, it's still having some limitations such as Physical aging effect, low crystallization rate, less thermal stability, and high production cost. Many strategies have been developed to tackle this issue by toughening of PHB through some techniques such as- Drawing and thermal treatment, Inclusion of natural fiber-reinforced composites- Natural and Inorganic fibers. The blending of PHB with natural, synthetic, or non-biodegradable polymers, and Chemical fictionalization. Toughening of PHB through the blending process: is the most effective and easiest way for making new materials having good qualities where different blends of polymers are used with PHB, e.g.- blends of starch, cellulose, chitin, etc, to enhance its function and increase its qualities (Yeo *et al*., 2018). All these processes are acquired for changing its physical properties, reducing brittleness and hardness, maintaining crystallinity or thermal properties and maintaining the aging effect and improving its qualities for better performance of PHB.

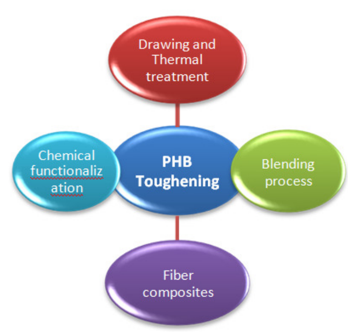


**Fig. 1.** Types of toughening processes.

**Applications of Polyhydroxybutyrate (PHB).** There are various important applications of PHB in different sectors. Since PHB is biodegradable and environment friendly and has very unique properties, it can replace various non-biodegradable plastics and materials that are causing harm to the surroundings. PHB can be used for several purposes because of its unique properties. In the table below, different properties and applications of PHB are mentioned.

**Table 3: Various applications of PHB and its properties.**

| Sr. No. | Different Industries | Applications | Unique Properties | References |
|---|---|---|---|---|
| 1. | Environmental | Biodegradable plastics,thermoplastics, disposable bags, bottles, etc | Biodegradability,eco-friendly, low thermal stability | (Sharma, 2019) |
| 2. | Pharmacology | Medicine encapsulation, controlled drug release | Biocompatibility,biodegradability, and non-toxicity | (Livshits *et al.,* 2009) |
| 3. | Medical field | Surgical implants, sutures, vascular grafts, or scaffolds | Biocompatibilityand non-toxicity | (Zaheer and Kuddus 2017) |
| 4. | Material packaging | Food packaging | Improved barrierperformance, UVresistance | (Bucci *et al*., 2005) |
| 5. | Agricultural field | Crop protection | Plant growth through bacterial support | (Alves *et al*., 2019) |

**Environmental applications.** The use of normal non-biodegradable plastics has created several environmental issues like soil pollution, water pollution, etc that have led to various harmful hazards. Since those regular plastics are non-biodegradable they stay as it is in the environment causing hazardous effects. To handle this huge problem and reduce the use of harmful and regular synthetic plastic in an eco-friendly way to protect the environment, PHB (Poly-hydroxy-butyrate) a bio-degradable bioplastic has emerged. PHB is been one of the most prominent bioplastics and can be used as an eco-friendly alternative to normal plastic and have more advantages than PE (polyethylene), PP (polypropylene), and other regular plastics because of its unique properties like having less permeability of PHB than these two plastics (Sharma., 2019). Due to their unique properties suchas fast degradability in the natural environment, high melting temperature, high crystallinity, and absence of residues unlike synthetic polymers, they are best for biodegradable plastic. Various industrial applications such as making disposable items, bags, bottles, etc have emerged. PHBs can degrade in any kind of environment like aerobic, anaerobic, or saline environment without providing any harm to the surrounding. During biodegradation in the aerobic environment, the end products are- $CO_2$ (carbon dioxide) and $H_2O$ (water) while the end product in the anaerobic environment is methane (Boey *et al*., 2021). Therefore it can be observed that PHBs are eco-friendly and easily degrades in the environment without causing any harm to the surrounding. Therefore it could be used as a biodegradable plastic instead of those synthetic plastics and could also be used for making disposable items such as bags, bottles, plates, plastics,etc.

**Applications in Pharmacology.** When any drug, tablet, or medicine is being prepared, its protection from the surroundings and controlled release is very important and must be considered. In Pharmacology, PHBs are used for encapsulation (protecting material from leaching out) of medicines, for controlled drug release, or as materials for cell and tablet packaging since they are biocompatible, non-toxic, and safer to use. They are used for protecting the tablets and medicines from getting leached out and protect it from the outside environment so that it will not get damaged by getting in contact with the toxins present in the surrounding. PHB is used for controlling the rate of a drug at which it is to be released into the system. It is important to maintain the drug level in the optimal range to maintain the drug level in the blood so that it will not do any harmful reactions inside the body. PHB-based microcapsules have numerous uses in the pharma industry such as the controlled release of water-soluble drugs, proteins, nucleic acids, and peptides. One of the examples of a PHB-based microcapsule is the methylene green drug. The biological and chemical nature of PHB makes it suitable for medical techniques and it also has the advantage of prolonged release of various drugs and makes it possible for controlled drug release (Livshits *et al*., 2009). Development of PHB base films and microcapsules for sustained drug delivery is going on. Scientists have developed systems of dipyridamole release based on PHB microspheres. Example- Sustained release of dipyridamole based on PHB microsphere occurs with a constant rate for more than 1 month. Moreover, PHB can be also used for producing enzyme activators and inhibitors for physiological model development (Bonartsev *et al*., 2007).

**Applications in the Medicinal field.** In the medicinal field, various necessary equipments and implants are required and made daily for the treatment. Many of them are dumped daily in the surrounding which could create a big issue for environment pollution if non biodegradable and hence it must be concerned. Therefore it is important for the implants should be made carefully so that it is safe and could be used without any danger and also will not create any environmental hazard. Since PHB is biodegradable and eco-friendly, it is used as an alternative to ordinary plastics because of its physical properties. There is a major application of PHB for development of medical devices such as screws and plates for bones and cartilage, and biodegradable sutures for dental implants, in facial and skin surgeries (Zaheer and Kuddus 2017).

These eco-friendly sutures and implants could be also used in tissue engineering. The PHB biocompatibility has been used for the growth of various cells, e.g. - Bone marrow cells, osteoblast, fibroblast, cartilages, etc. (Wani *et al*., 2016). PHB in contact with body fluids gets degrades into its monomers D, L- β – hydroxybutyrate and prevents apoptosis. They serve as outstanding innovations in different fields of medical science, biomedical engineering, and nanotechnology (Dalmia and Wadiye 2020). Since PHB is compatible with tissues and blood vessels of humans, the body could reabsorb PHB and it could be useful for surgical implants and as steam threads for healing wounds.

**Applications in Food Packaging.** Food packaging is a concerning topic since its protecting the food from the surrounding environment and microbes. Since the food particles are directly going inside our body, the packaging and total care and protection of food from the surrounding environment must be properly done. The common food packaging polymers are PE, PP, PET, PVC, etc. Innovation in food packaging increased due to the focus on the health and quality of food and also intend to reduce food preparation time and use of animal protein as well as concern about the environment (Khosravi-Darani and Bucci 2015). PHB can be used as a perfect alternative for food packaging due to its unique characteristics. In an experiment comparing PHB with PP (polypropylene) for packaging, it was observed that PHB showed a difference in deformation value which was 50% lower than PP value (Bucci *et al*., 2005). A comparison is done between the properties of PHB and PP in the given table for understanding their importance and efficiency:

**Table 4: Comparison of properties between PHB and PP (Markl *et al*., 2018).**

| Sr. No. | Properties | PHB | PP |
|---|---|---|---|
| 1. | Crystalline Melting Point(°C) | 175 | 176 |
| 2. | Molecular Weight in (Daltons) | $5\times10^5$ | $2\times10^5$ |
| 3. | Glass transition temperature (°C) | 4 | -10 |
| 4. | Material density | 1.250 | 0.905 |
| 5. | Tensile Strength | 40 | 38 |
| 6. | Ultraviolet rays resistance | Good | Poor |
| 7. | Solvent resistance | Poor | Good |

From the above table, in some cases, it was observed that some properties of PP were better than PHB and vice versa. It could be observed that PHB has good tensile strength and molecular weight and it can protect food protect for UV radiation more efficiently than PP. Through various research, it was concluded that PHB is safe to use in the future and PHB could be widely used for food packaging and could protect the food and maintain its quality.

**Applications in the Agricultural field.** PHB can be used in the agricultural industry to protect the crops from the soil pests and for encapsulation of fertilizers. It could be done through a "biofeedback" mechanism for agricultural extension (Holmes, 1985). PHB is an ideal bioplastic that helps in removal of waste without the useful chemicals being wasted. In the biofeedback mechanism, an insecticide is included with PHB granules sown with the plants in autumn. Bacteria start to colonies the PHB and insecticide is released after an induction period of a week. During winters when the temperature of soil decreases, microbial activities also gets reduced causing less release of insecticide and huge wastage of chemicals. Incorporation of PHB with the insecticide is that's why beneficial because as the pest activities also falls during winter, the active chemical is not being wasted. PHB metabolism contributes to the proper growth ability of H. *seropedicae*, a betaprotobacteria which performs nitrogen-fixation in various crops like maize *(Zea mays)*, rice (*Orzya sativa)*, pineapple *(Ananas comosus),* etc as biofertilizer for plant growth. PHB cycle ensures proper metabolic growth ability of bacteria to express its traits that will helpful for promoting plant growth and thebacteria which produces and store PHB have improved cell division and good stress tolerance level due to the energy released due to the PHB reserve mobilization (Alves *et al*., 2019).

Polyhydroxybutyrate (PHB) is an emerging biopolymer that could be produced from various cheap substrates and microbes. They are produced through some commonly used microbes such as *Cupriavidus necator*, *Bacillus subtilis* NG20, *Halomonas boliviensis, Bacillus megaterium,* etc., and through cheap substrates that are easily available. From the above article, it can be observed how PHB can be easily produced through low-cost substrates, solving the problem of production cost and other complications.

PHB has lots of advances and emerging applications in different industries and sectors. There are various future aspects of this biopolymer. It is used in various industries since it is non-toxic, biodegradable, eco-friendly, UV resistant, and safe. The problem of synthetic plastic could be solved by the increased production of PHB. Even in the present time, PHB is in great use in different industries such as in the pharma industry (Livshits *et al*., 2009), the medicinal industry (Dalmia and Wadiye 2020), the agricultural field (Alves *et al*., 2019), and is already replacing plastics and other polymers because of its unique properties (Sharma 2019).

In the upcoming future, PHB can reduce environmental pollution and will replace the use of synthetic plastic because of its unique properties and immense importance in various sectors mentioned above. Since PHB is a biodegradable and highly used polymer, there is no harm in using the biopolymer for various purposes.

## CONCLUSION

A review of various methods of production of Polyhydroxybutyrate (PHB) has been presented here, which has been mainly focused on its low manufacturing cost and environmental benefits. Several important aspects have been discussed, starting with the concern for energy and environmental sustainability and how PHB a plastic-like Biopolymer can be used as an alternative to synthetic plastic. Then the production of polyhydroxybutyrate by using cost-effective substrates and their strains such as sugarcane molasses, Canned pineapple (*C. necator* strain A04), Cow dung (*Bacillus pumilus* H9), Sugar industry wastewater, and Banana (*Musa* sp.) fronds extract (*Cupriavidus necator* H16) was drawn from different studies. The main hassle in the large-scale production of PHB using the physicochemical technique is the manufacturing cost as compared to the petrochemical-obtained plastic production.

Optimizing various inexpensive and natural substrates to overcome manufacturing costs in large-scale commercial production is one of the well-known trends in the industry today.

It was concluded that alkali pre-treatment and microwave pre-treatment are effective, energy-intensive, and simple methods for the optimization of PHB production.

Additionally, the trends in the recent development of PHB production through Cyanobacteria which have high protein content, Cupriavidus necator by using $CO_2$ as a renewable feedstock, Sugar refinery waste products, Polyacidic acid a biodegradable material originally used for food packaging, various Biocomposites used for making dental implants was observed. Furthermore, various important applications of PHB in different sectors including Environmental, Pharmacology, Medical field, Material packaging, and its unique properties such as biodegradability, low thermal stability, and non-toxicity came into view. In this sense, this biopolymer polyhydroxybutyrate is the most effective to reduce the pollution value of the environment, and in today's industry; there are various inexpensive substrates to overcome manufacturing costs in large-scale commercial production.

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