BIODEGRADABLE PLASTIC PRODUCTION FROM POLYHYDROXYBUTYRATE(PHB) PRODUCING BACTERIA AND ITS IMPACT ON ENVIRONMENT.

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Abstract

Plastic materials have been used in various ways such as packaging material, glass wares, certain parts of instruments of various grades, many more. As plastic products are not easily decomposed and accumulate in environment. This has created an adverse effect on our ecosystem. Bioplastics are best alternative to overcome these issues and reduce damages in ecosystem. Bioplastics are easily and speedily degrade under natural environmental conditions. Polyhydroxybutyrate or PHB is a bioplastic, one of the best natural polymer and alternative of synthetic plastic material. Bioplastic are comparative more flexible to convert into various forms also. Normally, it is a lipid reserve material produce in adverse or under stress conditions and stored within the cell wall of micro-organisms. Poly-3-hydroxybutyrate (PHB) is a natural biopolymer produce by wide range of microbes which is a storage granule used by microbes as an energy as well as carbon source. this microbes includes from extremophiles to mesophiles. The objective of our work was to isolate potential PHB producing bacteria using agro-residues as carbon sources within natural environment conditions. This work describes the understanding of microbes that are involved in the degradation of both natural and synthetic polymers. Also to contribute in green environment by providing eco- friendly product.

Keywords: Polyhydroxybutyrate, Ecosystem, Bioplastics, Eco-Friendly Product

INTRODUCTION

Natural polymers (from the Greek poly meaning “many” and meros meaning “parts”) are found in many forms such as horns of animals, tortoise shell, shellac, rosin (from pine trees), asphalt, and tar from distillation of organic materials. One of the most useful of the natural polymers was rubber, obtained from the sap of the hevea tree. Rubber was named by the chemist Joseph Priestley who found that a piece of solidified latex gum was good for rubbing out pencil marks on paper. In Great Britain, erasers are still called “rubbers”. Natural rubber had only limited use as it became brittle in the cold and melted when warmed. In 1839, Charles Goodyear discovered, through a lucky accident, that by heating the latex with sulfur, the properties were changed making the rubber more flexible and temperature stable. That process became known as vulcanization (Katz, D.A., 1998). The first synthetic polymer, a

phenol-formaldehyde polymer, was introduced under the name “Bakelite”, by Leo Baekeland in 1909. Its original use was to make billiard balls. Rayon, the first synthetic fiber was developed as a replacement for silk in 1911 (Armitage et al., 1973).

Since synthetic plastics marked their introduction in the 1950s, they have emerged to be among the most needed material in our daily life. These petroleum-based plastics are very stable in harsh conditions especially against the attack of chemical degradation and microbial decomposition, thus rendering them durable, highly resistant and ‘blessed’ with a very long life span in the environment. Due to their excellent properties and wide range of application, synthetic plastics have since championed the commodity market and the technologies related to plastics manufacturing are very well established. In fact, it is impossible to imagine everyday life without synthetic plastics.

However, most of these plastics utilize fossil carbon, especially raw petroleum as the starting material and eventually accumulate in the environment when discarded. The accumulation of plastic wastes is evident all over the world where natural ecosystems such as rivers, lakes and mangroves are polluted with these recalcitrant materials. In the tropics, discarded plastics pose an additional problem; they can become excellent breeding locations for mosquitoes when the discarded plastics contain moisture. To overcome issues of pollution and imbalance of nature due to synthetic polymers gradually we need to replace it with biopolymers.

Biopolymers (Bioplastic):

Thermoplastics produced from renewable agricultural and forest resources (biomass), also called bioplastics, are gaining in importance. For this study, we are investigating the opportunity for biobased thermoplastics in the disposable plastics market segments as opposed to engineering thermoplastics or thermoset plastics/ resins.

In order for a bioplastic to be considered compostable it has to satisfy three criteria:

- 1) Biodegradation – breaks down into carbon dioxide, water and biomass at the same rate as cellulose
- 2) Disintegration – the plastic is indistinguishable in the compost from other biomass material after a fixed schedule of time
- 3) Non Toxic – the residual biomass material must not be harmful to animals or plants in final form

They are polyesters obtained by polymerisation of monomers prepared by fermentation process (semi-synthetic polymers) or produced by a range of microorganisms, cultured under different nutrient and environmental conditions (microbial polymers) (Madison and Huisman, 1999). These materials are accumulated in microorganisms as storage materials.

Most of our study deals with Bacterial polymers of poly(hydroxyalkanoates) (PHAs). the properties of PHB as a biodegradable thermoplastic material have captured attention for more than 20 years, and patents were originally filed in the United States by J. N. Baptist in 1962, the first industrial production of PHB and PHA did not occur until 1982, when ICI plc marketed them under the trade name Biopol. Marchessault et al., (1988) have also drawn attention to the potential role of PHAs as so-called biomass transducers, i.e., their use in the microbial transformation of carbohydrate feedstock via PHA into chiral depolymerization products

(Seebach and Zuger, 1982), (Seebach and Zuger, 1985) or small molecule organic chemicals by pyrolysis.

The current sharp focus of attention on the environmental pollution caused by discarded petrochemical plastics has given added force to studies of the biodegradation of PHAs in the natural environment. However, against these important applied aspects there has also been an increasing appreciation of the role of such polymers in the life of microorganisms in their natural habitats.

A number of bacteria can accumulate PHAs as intracellular reserve materials. Some organisms accumulate PHA from 30% to 80% of their cellular dry weight, in the presence of an abundant source of carbon and under limited nitrogen (Mercan et al., 2002).

Chen and Wu (2005) reported that Polyhydroxyalkanoates or PHAs are linear polyesters produced in nature by bacterial fermentation of sugar or lipids. They are produced by the bacteria to store carbon and energy. More than 150 different monomers can be combined within this family to give materials with extremely different properties. These plastics are biodegradable and are used in the production of bioplastics. They can be either thermoplastic or elastomeric materials, with melting points ranging from 40 to 180 °C. The mechanical and biocompatibility of PHA can also be changed by blending, modifying the surface or combining PHA with other polymers, enzymes and inorganic materials, making it possible for a wider range of applications.

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoates (PHA), a polymer belonging to the polyesters class that was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne. PHB is produced by microorganisms (like *Ralstonia eutropha* or *Bacillus megaterium*) apparently in response to conditions of physiological stress. The polymer is primarily a product of carbon assimilation (from glucose or starch)

MATERIALS AND METHODS

Sources:

Soil Samples were collected from different agriculture fields surrounding Kalol, Certain samples were also taken of raw milk from local dairies.

Isolation:

Serial dilution of each sample was done to obtain well isolated colonies upto 10^{-9} . Spread plate technique was used to isolate microbes. All the prepared diluted samples were spreaded on Nutrient agar plates in duplicates and incubated for 24-48hours at 28-30°C.

Identification:

Well-isolated colonies were picked up and gram-staining was performed to check their types and purity. Furthermore bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black B dye (Juan et al., 1998). The dark blue coloured colonies were taken as positive for PHB production.

Colony characteristics

Colony characteristics like size, shape, margin, elevation, texture, consistency, opacity and pigmentation were considered for the primary identification of the bacterial isolates capable of producing PHB

Confirmation of PHB producing bacterial isolates by PHB staining

All bacterial isolates were stained by Sudan Black B staining method (Byrom and Byrom, 1991).

Method of staining PHB granules

1. Spread the culture on a clean, sterile, grease-free slide, using the nichrome wire loop. The smear should cover an area about 10 mm x 30 mm.
2. Allow the smear to dry in the air.
3. Fix the smear by holding the slide with forceps and passing it horizontally through a small Bunsen flame 2–3 times.
3. Fixing kills the bacteria by coagulating the cytoplasm. It also sticks them to the slide.
4. Place a few drops of Sudan Black solution on the fixed preparation. After 5–10 minutes the ethanol in the stain should have evaporated. Any excess liquid can be carefully drawn off using the edge of a piece of filter paper.
5. Immerse the slide in xylene until it is completely decolorized (this takes about 10 seconds). Allow the slide to dry
6. Flood the slide with the counterstain, Safranin solution for 10 seconds, gently rinse the slide with running water.
7. Allow to air dry the slide completely and examine with an oil immersion lens. The PHB can be seen as very dark granules inside pink cells

Standard Curve For PHB

Pure PHB was used to obtain standard curve using Law and Slepecky method given in 1961. 1 mg was dissolved in 10 ml of concentrated H₂SO₄ and heated for 10 min to convert PHB into crotonic acid, which gave 0.1 mg/ml concentration.

Polymer (PHB) Extraction. (Law and Slepecky, 1961)

1. For the assay of polymer in small quantities of cells, the organisms were centrifuged in polypropylene centrifuge tubes which had been previously washed thoroughly with ethanol and hot chloroform to remove plasticizers.
2. The cell paste was resuspended in a volume of commercial sodium hypochlorite solution (Clorox or equivalent) equal to the original volume of medium.
3. After 1 h incubation at 37° C, the lipid granules were centrifuged, washed with water followed by washing with acetone and absolute alcohol.
4. Finally, the polymer was dissolved by extraction with three small portions of boiling chloroform, the chloroform solution was filtered, and the filtrate was used for poly-β-hydroxybutyrate assay.

Spectrophotometric Assay

1. A sample containing extracted polymer in chloroform is transferred to a clean test tube.
2. The chloroform is evaporated and 10 ml of concentrated H₂SO₄ is added, the tube is capped with a glass marble and boiled for 10 min at 100° C in a water bath.
3. The solution is cooled, and after thorough mixing, a sample is transferred to a silica cuvette and the absorbance at 235 nm is measured against a sulfuric acid blank.

The most potential bacterial strain was selected which showed highest PHB production determined by the spectrophotometric assay.

Identification

Morphology of the most potential strain producing PHB was determined by Monochrome staining & Negative staining technique. Also, Gram staining technique was used to check its Gram's reaction. Spore staining was carried out by Bartholomew and Mittwer's method.

while Biochemical characterization of the strain was carried out by using various tests like fermentation of sugars, indole production, methyl red, voges-proskauer, citrate utilization, nitrate reduction, ammonia production, H₂S production, lipid hydrolysis, starch hydrolysis, gelatin hydrolysis, urea hydrolysis, dehydrogenation and catalase test. Biochemical tests were carried out as per the method given by (Cappuccino and Sherman, 1992) with 24 h old culture.

RESULTS AND DISCUSSION

PHB is accumulated by numerous microorganisms and is the best characterized PHA (Madison and Huisman, 1999). 12 different soil samples and 4 raw milk samples were used to obtain desired bacterial isolates. serial dilution upto 10^{-9} was useful to obtain isolated colonies with desired properties.

All spreaded plates from various diluted samples were incubated for 24-48 h which yielded maximum and well isolated young growing bacterial colonies. Those colonies were very easily understood for different characteristics.

Colony Isolation and characteristics

PHAs, synthesized by many Gram positive and Gram-negative bacteria as storage compounds, are deposited as insoluble inclusions in the cytoplasm (Steinbuchel, A., 1991). Hartman, T.L. (1940) was the first to suggest the use of Sudan black B, as a bacterial fat stain.

Majority of isolated colonies with desired properties belongs to *Azotobacter*, *Bacillus*, *Pseudomonas* and *Archeabacteria* species. All of them have different ratio of PHB accumulation. *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) has been the subject of much published research work because it can accumulate PHAs up to 80 per cent dry weight (Lee, S.Y., 1996). All isolates with desired properties were coded PH1 to PH8. Majority of isolates belongs to *Bacillus strains*, *Azotobacter strains*, *Micrococcus strains* and *lactobacilli strains*.

CONCLUSION

In the present study, an attempt was made to isolate efficient PHB producing bacteria from samples collected from various sources like soil, water and milk. With a concern to make product economically feasible, cultural parameters like media components and also growth conditions were optimized for the most potential bacterial isolate. Various diluted samples of soil, water and milk were used to isolate PHB producing bacterial strains primarily with the help of Nutrient agar medium because it favours the growth of diverse group of bacteria.

Primary screening of PHB producing bacterial colonies was carried out by using viable colony method with the help of Sudan Black B solution. Total 08 different types of colonies were found as PHB positive strains by using viable colony method. Secondary screening was done

with the help of Sudan Black B staining and Quantitative PHB determination by using UV Spectrophotometric assay. All the isolates were showing presence of PHB granules. Different groups of bacteria like, *Bacilli*, *Azotobacter* and *Micrococci* were observed as dominant PHB producers among the isolated strains. One bacterial strain, *Bacillus* PH5 coded as was found to produce highest PHB among all the other isolated PHB producing bacterial strains. *Bacillus* PH5 was observed to produce 48 % by using Nutrient broth.

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Table 1 Colonial characteristics of PHB producing bacterial isolates

Colony	Size	Shape	Texture	Consistency	Opacity	Margin	Pigmentation
PH1	Small	Round	Smooth	Moist	Opaque	Even	Light Yellow
PH2	Intermediate	Round	Smooth	Moist	Opaque	Even/Entire	Reddish
PH3	Small	Round	Smooth	Moist	Transparent	Even	Nil
PH4	Intermediate	Irregular	Smooth Glistening	Moist	Translucent	Rhizoid, Uneven	Dull White
PH5	Big	Round	Smooth	Butyrous	Opaque	Uneven	Off White
PH6	Intermediate	Irregular	Rough	Dry	Opaque	Uneven	Nil
PH7	Big	Round	Rough	Dry	Opaque	Undulate	Nil
PH8	Small	Round	Smooth	Moist	Opaque	Entire	Nil

Standard graph of PHB UV-Spectrophotometric assay

Table 2: Dry Cell Weight and PHB production in N-broth by selected strains after incubation of 48 h at 30° C.

Isolates	Probable strain	Dry cell weight(g/l)	PHB(g/l)	PHB%
PH1	Azotobacter	0.826	0.313	31
PH2	Micrococcus	0.761	0.106	17
PH3	Micrococcus	0.553	0.209	21
PH4	Bacillus	0.451	0.089	10
PH5	Bacillus	0.813	0.489	48
PH6	Bacillus	0.713	0.239	26
PH7	Bacillus	0.745	0.110	19
PH8	Azotobacter	0.859	0.473	43

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