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A STUDY ON BIOPLASTICS PREPARATION USING BACTERIAL POLYHYDROXY BUTYRATE AND BIODEGRADATION

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Abstract

Plastic has become an inevitable material to mankind and hence in use for hundreds of years. But the impact of plastic pollution is a serious threat to the ecosystem as well as to the atmosphere. Plastics need to be replaced with the environmental - friendly biodegradable bioplastics. Polyhydroxybutyrates are the promising bioplastics which are produced by plant sources as well as microbial sources. The current research focuses on bacterial based polyhydroxybuutyrates. Polyhydroxybutyrates can be used as an alternative to conventional plastics and is currently now applied in medical field and tissue engineering applications. Polyhydroxybutyrate producing bacteria were screened and isolated from agricultural soil sample through sudan black blue staining. Large quantities of PHB are produced in minimal essential medium with glucose as the chief carbon source. Bioplastics are prepared through solvent casting technique. To overcome the brittle nature of PHB, they are blended with starch and glycerol. Nitric acid based chemical degradation was done and it was found to be efficient with a gradual decrease in the weight of PHB as the concentration of nitric acid increases. Biodegradation of produced PHB was done with *Pseudomonas* and *Bacillus species* and halozone formation was seen in petriplates indicating biodegradability. When degraded under natural soil environment, weight reduction was noted with an overall 88 % weight loss. Thus, PHB is biodegradable and can be used as efficient bioplastics.

Keywords: Polyhydroxybutyrates, Sudan black blue staining, bioplastics, biodegradability, solvent casting technique.

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Introduction:

Plastic pollution has an adverse effect on the environment, human life, animals, agricultural fields, and other areas. Plastics production (450 million tonnes per year globally), consumption, and recycling (300 million tonnes per year internationally) are among the most serious environmental challenges and global impediments to waste management practices (Hakuzimana *et al*., 2017). According to projections, annual garbage creation would rise by 70% due to population expansion and urbanisation from 2016 levels to 3.4 billion tonnes in 2050. Plastic debris has impacted both inland and aquatic habitats, prompting scientists to speak out on several times in recent decades (Dacosta *et al.*,2020). Plastics have a negative impact on wildlife, flora and fauna, soil, waterbodies, and, eventually, human existence. There is a demand for bioplastics that are totally biodegradable and environmentally beneficial.

Poly ethylene (PE), Poly Ethylene Terephthalate (PET), Poly Styrene (PS) and Polypropylene (PP) currently account for 60% of the gross plastics market in developing nations. Biodegradable polymers such as Poly Hydroxy Alkanoates (PHAs), Poly Lactic Acid (PLA), and Poly Butylene Adipate-co-Terephthalate (PBAT) are well-established in the market.

Lemoigne first discovered polyhydroxybutyrate (PHB), a well-studied type of polyhydroxyalkanoate, in 1925 (Lemoigne *et al*.,1995). PHB is a biodegradable plastic with a Molecular Weight (MW) ranging from 1x104 to 3x106 Da (Macrae *et al*., 1958). The glass transition temperature, crystalline density, and amorphous PHB are 180°C, 1.26, and 1.18 g (cm3)-1, respectively (Ray *et al*., 2017). PHB has a lower extension to break (5%) than Polypropylene (400%) despite having identical tensile strength and Young's modulus (Khanna *et al*., 2017, Koning *et al.*, 1992).

Because they are biodegradable and generated from microorganisms (Anjana *et al*., 2021) polyhydroxy butyrates (PHBs) are promising bioplastics. Microbial fermentation produces PHB, which is then processed by microbial cell lysis. PHBs are compostable, non-toxic biopolymers produced from (R)-3-hydroxyalkanoic acids (Sudesh *et al*., 2000), having thermoplastic characteristics equivalent to crude oil-based plastics (Penkhrue *et al*., 2020).

Working with soil bacteria is unique in that it has a vast spectrum of potent strains capable of creating a variety of enzymes, metabolites, antibiotics, antioxidants, inhibitors, and other substances. Biopolymers can be synthesised by more than 80% of soil bacteria. Because it can collect PHB on its own, Ralstonia eutropha is a model organism for PHB production (Anish *et al*., 2013, Mahitha *et al.*, 2023). Many scientists have documented PHB production by *Bacillus species, Alcaligenes species, Azotobacter species, Nocardia species, Pseudomonas species* and *Rhizobium species.*

In the current research conducted, agricultural soil sample was taken as the source and from it PHB producing Bacteria were screened and isolated. The PHB were produced in large quantity and extracted through sodium hypochlorite method. In order to prepare bioplastics, PHB should be prepared in large quantity. To increase the plasticity and overcome the brittle nature of PHB it was blended with starch. To prove it as ecofriendly and biodegradable it was checked for Biodegradability in lab as well as in natural conditions.

Methodology:

Sample collection:

Soil sample for the research was collected from an agricultural land located at Devasahaya nagar, nattalam, Kanyakumari district, Tamil nadu. The soil

was collected from a depth of 10cm in a sterile container and transferred immediately to the laboratory.

Serial dilution, plating technique:

The soil sample was serially diluted upto 10^{-10} dilution and plating technique was performed to isolate individual colonies of bacteria. Further, the plates were checked for sudan black blue staining.

Sudan black blue staining in petriplates:

For this experiment, 0.9% sudan black blue stain was prepared in absolute ethanol. The culture plates were flooded with sudan black blue stain and left untouched for about 20 minutes. After 20 minutes the excess dye was poured out and 80% ethanol was used to wash away the unbound stain. If necessary, the plates can be washed again. The colonies which acquired the stain and appeared bluish black are positive isolates and those which did not acquire the stain and appeared colourless are negative isolates. (Macrae *et al.,* 1958)

The positive PHB producers were sub streaked immediately for further use. Out of the numerous strains stained bluish black, five colonies were randomly selected and named as SM16, SM 17, SM 18, SM 19 & SM 20.

Microscopic observation of PHB positive isolates:

To confirm the existence of PHB granules in the cytoplasm of bacteria, 0.3% sudan black blue stain was used. The prepared stain was filtered to remove the sediments. The bacterial strains were smeared on a clean glass slide and heat fixed. Few drops of stain was poured over the smear and allowed to stand for about 20 minutes. It was then counter stained with few drops of safranin for about 10 to 20 seconds, air dried and viewed under microscope. (Macrae *et al.,* 1958)

PHB production media:

The PHB producing bacteria were inoculated in PHB production media containing the ingredients like, Glucose – 40g/l, Urea – 1g/l, Yeast extract – 0.16g/l, Potassium hydrogen phosphate-1.52g/l, Disodium hydrogen phosphate-4g/l, Magnesium sulphate-0.52g/l, Calcium chloride-0.02g/l. Trace element solution are also added to production media in smaller quantities which includes, Zinc sulphate-0.13g, Ferrous sulphate-0.02g, Ammoniummolybdate-0.06g, Boric acid-0.06ml

Extraction of Polyhydroxybutyrate:

Polyhydroxybutyrate extraction from the specified bacteria was achieved through sodium hypochlorite method which is as follows: The five indigenous species were cultured in nitrogen-deficient but carbohydrate-sufficient minimal essential medium and incubated for approximately 72 hours. PHB extraction was carried out in accordance with previous research. Following incubation, the cultures were centrifuged for about 10 minutes at 10,000 rpm to obtain the culture filtrate. The supernatant was discarded, and 10ml of sodium hypochlorite was added and thoroughly mixed into the pellet. It was then incubated for about an hour in a water bath kept at 50^{o}C. After one hour, it was centrifuged for 15 minutes at 5000 rpm. The pellet was then washed with distilled water, acetone, and ethanol. 5ml of boiling chloroform was used to dissolve the pellet. The contents were left to evaporate overnight in a sterile glass plate at 4 °C. The powdered PHB settled in the glass plate after evaporation of chloroform. It was collected with a sterile spatula as and stored in sterile glass bottles for further analysis. Based on the amount of PHB produced, the best PHB producing bacteria was identified.

Bioplastics preparation:

Among the isolated cultures, the potent PHB producer was used for Bioplastics or in other term biofilm preparation. Various combination of PHB mixture are used for this process. Bioplastic film was developed by mixing 50 mg of

PHB in 10 ml of chloroform in a beaker. The mixture was stirred well. It was slightly heated for complete mixing of PHB. After this, the mixture was poured on petriplates or aluminium foil and allowed to dry. It was allowed to dry for about two days. After that, the obtained biofilm was used for other studies (Rawia *et al*., 2013).

In another method, 250 mg of PHB were dissolved in 28 mL of chloroform. The solution was evenly divided among five petri dishes. The dishes were kept at 30 degrees Celsius to allow the chloroform to completely evaporate. The formation of PHB films in the petri dishes was caused by the evaporation of the solvent. Vacuum drying was then used to completely remove the remaining solvent from the films (Kai *et al.*, 2003).

Blending of PHB:

In order to overcome the brittle nature of PHB plastic film, blending agents, Plastictisers can be added to the mixture to increase the plasticity.

Blending with starch:

Various concentrations of PHBs and commercial starch in the ratio of 90:10, 80:20, 70:30, 60:40, 50:50 were prepared. To that 2ml of glycerol was added which act as a plasticiser. Then, the mixture was evenly distributed in the petriplates and allowed to dry for 48 hrs. After 48 hrs dried biofilm or bioplastic of PHB are obtained.

Natural starch like potato starch and corn starch were also utilised for Bioplastics preparation. They were mixed with PHB and few drops of glycerol were added to enhance the bioplastics preparation. Each time when PHB was used for bioplastics preparation , they are dissolved in chloroform through gentle heating.

Degradation studies:

Chemical degradation of extracted PHB powder by nitric acid:

Nitric acid was used to degrade the bioplastic films made from the derived PHB granules at various concentrations (0.2, 0.3, 0.5, 0.8, and 1%). PHB powder was spread on the X-ray films. The initial weight of PHB powder was measured. At regular intervals, varying concentrations of nitric acid were sprayed on PHB. The final weight was calculated after degradation.

Biodegradation studies through halozone formation:

Bacillus species and *Pseudomonas species* were used to test the biodegradability of the developed plastic sheets. The nutrient agar plates were prepared and a cavity was formed by gel puncturing at the center. PHB was dissolved, and 100 ul of it was poured into the center cavity. The entire petriplate was then inoculated with *Pseudomonas species*. It was then incubated for 24 - 72hrs at 37ºC in an incubator. The petriplate was checked for the presence of a hallow zone after 24 hours (Kritika *et al.*,2015)

Biodegradation of PHB in natural environment:

The degradation of PHB in natural environment was checked in soil environment. Garden soil was collected in pots and kept under sunlight. The initial weight of the biofilm was recorded. The biofilm was buried into the soil environment at the centre of the pot. It was kept undisturbed for few days. Every seven days, the biofilm was taken out and it's weight was recorded. This step was repeated upto 45 days and weight of the biofilm was recorded. The percentage of degradability was measured using the following formula. (Bano *et al*., 2019)

$$\% \text{ Degradation} = \left[\frac{(W_{if} - W_{ff})}{W_{if}}\right] \times 100$$

where, W_{if} = initial weight of films,

W_{ff} = final weight of buried films.

Results and Discussion:

Soil sample is an inhabitant of many beneficial microbes especially bacteria. In the current research, agricultural soil sample was taken from 10cm soil profile where most of microbial diversity is present and microbial population is actively concentrated. From it, 1g of soil sample was serially diluted and sudan black blue staining was performed. Sudan black blue staining helps in identification of PHB positive isolates. More than 90% of bacteria stained positive for sudan black blue staining (fig 1). Out of it five prominent individual colonies were randomly selected. The strains were designated as SM16, SM17, SM18, SM19, SM20. They were substreaked again and confirmed for PHB with sudan black blue staining. The PHB granules in their cytoplasm were visualised under microscope (fig 2). The PHB granules were observed as distinct granules and distributed throughout the cytoplasm. The strains were maintained as slants and stored for further use.

All the five strains were further cultured repeatedly for PHB production to obtain more quantities of PHB. Production medium contains the following ingredients: Glucose, Urea, Yeast extract, Potassium hydrogen phosphate, Disodium hydrogen phosphate, Magnesium sulphate, Calcium chloride and Trace element solution like, Zinc sulphate, Ferrous sulphate, Ammonium molybdate, Boric acid. Extraction of PHB was done through sodium hypochlorite method. Among them, the best PHB producer was found to be SM20 through cell dry weight method and used for further studies.

PHB are produced in large quantities by repeated culturing in production medium with glucose as the sole carbon source. PHB production is better achieved in medium with high carbon source and low nitrogen source. Carbon nitrogen ratio plays an important role in PHB production. Gomez and Getachew reported that when more carbon sources were added to the medium, the Phb yield increased steadily, indicating that the Bacteria also rely on glucose for energy production. Adwitiya reported that when glucose was used as the sole carbon source, the yield of PHB was more.

Conventional solvent casting technique is used for biofilm or bioplastic preparation as described by (Sridewi *et al*., 2016). The produced PHB powder should be dissolved in chloroform by gentle boiling to do biofilm preparation. Chloroform is the best solvent for dissolving the PHB. Other solvents include hexane, acetic acid, propanol, DMSO, Methanol, ethylene carbonate, Dimethyl formamide (Aramwash etal., 2017). The produced PHB is more of crystalline and brittle in nature indicative as in fig 3. So, after drying, they were not able to form a smooth plastic sheet. There were many holes and breaks in the plastic sheet. When removed from the petriplate or aluminium foil they were easily broken down. Atlae etal., in 2016 reported that when PHB sheets were observed over a period of time, cracks, holes, colour changes, gradual loss of components were observed. To overcome this problem the PHB powder can be blended with blenders and plasticisers. On addition of these additives the plasticity and elasticity nature of the bioplastic sheet increases.

To achieve bioplastics preparation starch can be added as the blender (Zhang *et al*., 2010). PHB and starch were added in the ratio of 70:30. To enhance the plasticity a small amount of glycerol can be added to the mixture. The blended bioplastics films (fig 4) and dried bioplastic sheets (fig 5) were picturised. A study by Reis et al.,2008 indicated that in FTIR measurements there was no intermolecular interaction between the two polymers as there was no shift in the absorption peaks of PHB-HV or starch blends were noted. Natural starch like corn starch and potato starch were also used for bioplastics preparation. This would make the bioplastics preparation cheaper and more natural. Certain natural colour

additives can also be added to this to make the prepared bioplastics colourful.

To know whether the produced PHB can be degraded it was checked for chemical degradation under laboratory conditions. Different concentrations of nitric acid 0.1, 0.3, 0.5, 0.8 and 1% were prepared and used for this process. PHB powder was used for this chemical degradation. There was gradual weight loss in the PHB as the concentration of nitric acid increases as indicated in table 1. Higher weight loss was noted with 1% nitric acid with a loss of 1.5 grams. These results indicate that the produced PHB are liable to change and can be moulded to any forms and they are degradable under laboratory conditions. This is in contrast to conventional plastics, which are non-polar materials like oil and they do not react with acids. The plastics are highly resistant to acids because they are polymers which do not form ions and they are held together by vanderwalls forces. (Thomson *et al*., 2009)

The ultimate objective of bioplastics would be the ability of biodegradation. Biopolymers are capable of degradation by microorganisms like bacteria. Under in vitro conditions, the biodegradation is checked with the halozone formation in petriplates. Produced PHB powder was mixed with media and poured into petriplates with small punctured wells at the centre. Just 100ul of bacteria was poured at the centre and the plates were incubated for 24 – 72 hrs. Mona *et al*., 2001 interpreted the biodegradation of the PHB by clear zone formation by the strain S. sp. SNG9 during 72 hours of incubation. If the bacteria are capable of degrading PHB it will form a halozone around the cavity. When *Bacillus species* was used, it showed smaller halozones (fig 6.b), indicating the ability of mild degradation. When *Pseudomonas species* was used, it showed a prominent, distinct halozones (fig 6.a) indicating the capability of potent degradation. Thus, *Pseudomonas species*, a universal biodegrader was capable of efficient degradation of PHB and can be used in future. Phb depolymerse enzymes present in the bacteria are helpful in PHB degradation. Because of its significance in biodegradable plastic degradation, this natural enzyme is gaining commercialization attention.

The produced biofilm was dug under soil taken in pot, for 45 long days it showed a considerable decrease in weight loss. The initial weight of the biofilm was 4.5g and at the end of 45 days the weight of the biofilm was 0.5g as indicated in table 2. So, the overall weight loss percentage was 88.88% which was a notable decrease in weight loss when compared with conventional plastics. The prepared bioplastics are completely degradable at the end of 60 days and there were no traces seen. Reports indicated by Amir *et al.*, 2022 indicate that soil-buried PHB polymer film was removed from soil pots after 15, 30, and 45 days of incubation. It was discovered that the highest microbial biodegradation occurred after 45 days of incubation. PHB film weight loss after 45 days of incubation was 94.4% after the soil burial approach, which is 8.64% higher under optimised settings than under unoptimized conditions. This demonstrates that the prepared bioplastics are degraded under natural soil environment and safer to use.

Conclusion:

The produced polyhydroxybutyrates can be efficiently used as bioplastics. However, to maintain the plasticity and make the bioplastics mouldable into any form, it can be blended with small amount of starch. Biodegradation of these bioplastics are efficient when checked under in vitro condition and under natural soil environment. So, Polyhydroxybutyrates can be used as an alternative to conventional polypropylene based plastics. To replace they should be produced in large amount in fermenters at industrial scale.

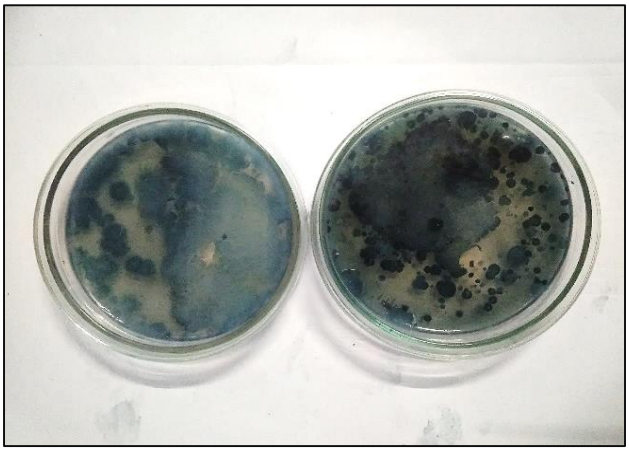


Fig 1: Sudan black blue staining in petriplates

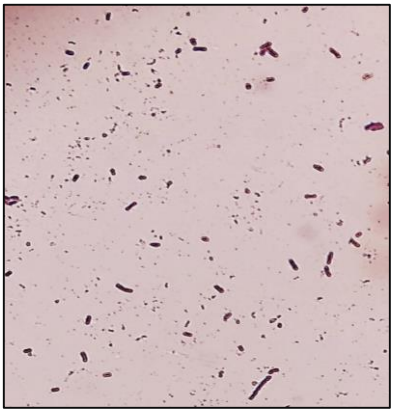


Fig 2: Microscopic observation of PHB positive colonies

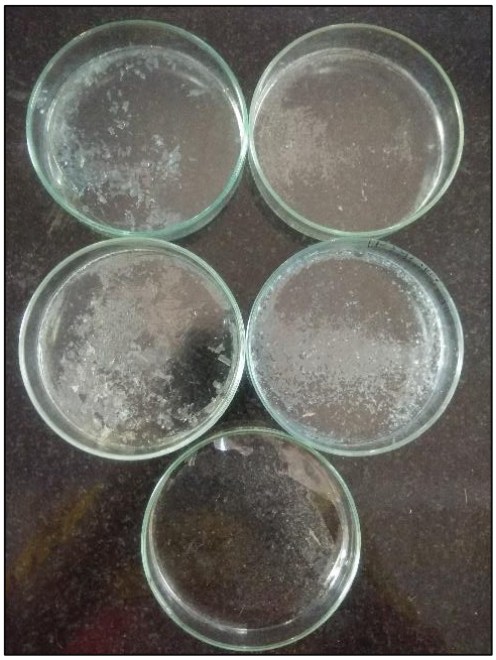


Fig 3: Bioplastics preparation of PHB without blending

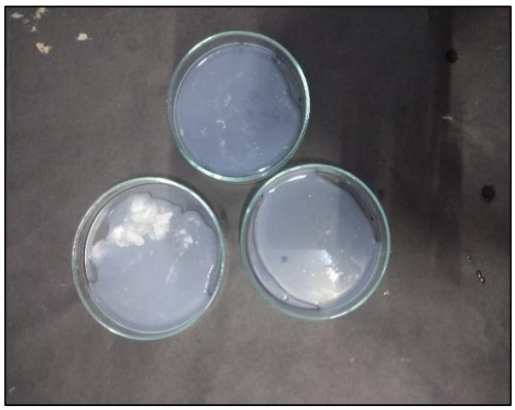
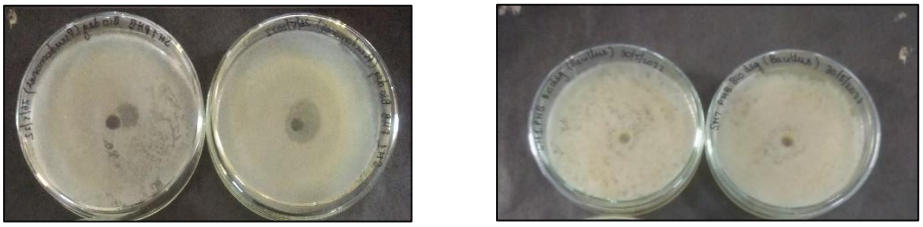


Fig 4: Bioplastics preparation of PHB by blending of starch

Fig 5: Dried PHB bioplastic sheets



A B

Fig 6: Biodegradation of PHB powder A) using *Pseudomonas species*
B) using *Bacillus species*

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Concentration of nitric acid (%)	Weight of PHB crystals on X-ray sheet (gm)		PHB degradation (gm)
	Before application	After application	
0.1	5	4.8	0.2
0.3	5	4.4	0.6
0.5	5	4.2	0.8
0.8	5	3.9	1.1
1.0	5	3.5	1.5

Table 1: Degradation of PHB with nitric acid

Table 2: Biodegradation of PHB in natural soil environment

Time period	Initial weight of biofilm (g)	Final weight of biofilm (g)	Percentage of biodegradation (%)
7^{th} day	4.5	3.9	13.33
14^{th} day	4.5	3.4	24.44
21^{st} day	4.5	2.8	37.77
28^{th} day	4.5	2.1	53.33
35^{th} day	4.5	1.4	68.88
42^{nd} day	4.5	0.8	82.22
45^{th} day	4.5	0.5	88.88

Statements & Declarations:

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Contribution by authors:

All the authors contributed equally to this research work, including conceptualization, design and discussion. Data collection and experimental procedures, were carried out by Ms. S.Mahitha. Data analysis, interpretation of results, discussion and conclusion were collectively done by all the authors.

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