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Biotechnology

BIOLOGICALLY SYNTHESIZED PLASTIC MATERIAL [POLY-ß-HYDROXYBUTYRATE (PHB)] FROM CHLAMYDOMONAS GLOBOSA

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ABSTRACT In today's world of science, plastic materials are used widely all over the world. The uncontrolled utilization of plastics leads to the major cause of waste accumulation on landfills and also the release of greenhouses. A biologically synthesized plastic material namely Poly-ß- hydroxybutyrate (PHB) is of significant interest because of its mechanical and physical properties similar to conventional plastics produced from petroleum products. PHB based plastic substitutes are found to be less flexible than traditional plastics and they are found to be completely biodegradable and leaves behind no residue.1 Algae are used for the production of PHB, for bioplastic production which offers an excellent opportunity in economic efficiency by reduced costs. Chlamydomonas globosa was isolated from freshwater sources and screened for PHB production using Sudan black B and Nile Blue A Stain. Various inexpensive substrates like Rice boiled water, Potato boiled water, etc has been used for the production of PHB. Hot chloroform was used to extract PHB and the amount of PHB produced was estimated by reading the absorbance at 235 nm. The extracted PHB was characterized by FTIR and NMR. It was further blended with other polymers like PLA to improve its physical characteristics. The thermal properties of Polymer were studied using Differential Scanning Calorimetry (DSC), Thermo gravimetric Analysis (TGA), Powder X-ray Diffractometer (XRD).1 The polymer shows good tensile strength with a low extension to break ratio comparable to petrochemical plastic. Applications of PHB include Shelf Life Testing, Migratory Tests, and the production of various biodegradable products like small covers and pouches.

KEYWORDS : Biodegradable – Biopolymers – Inexpensive Substrates - Nile Blue – Petrochemical – PHB

INTRODUCTION

Polyhydroxy butyrate (PHB) is completely biosynthetic and biodegradable with zero toxic waste and completely recyclable into organic waste. It was first discovered by M. Lemoigne in the year 1926. The superior characters of plastics are its strengths, durability, moldability, and shape which makes mankind to depend on plastic for their daily life. Since these plastics have high molecular weight and tightly bonded together, they are non-biodegradable and this will make their disposal difficult which inversely leads to negative impact on the environment. Petroleum-based Plastics are resistant to attack by harsh degrading chemicals and microbial depolymerases of naturally occurring soil bacteria. Therefore, disposing them in the environment results in pollution of the ecosystem.

Many different types of bacteria and algae produce PHB as food storage material.[2] Biodegradable plastics can decompose into carbon-dioxide, methane, water, inorganic compounds or biomass via microbial assimilation.[3] PHB has thermoplastic process ability, non-toxicity and high crystalline features. It is also a water-resistant polymer and completely biodegradable.[4]

Algae can be used as an excellent feedstock for bioplastic production[3] owing to its many advantages such as high yield and the ability to grow in a range of environments.[5] Bioplastics from algae are referred to as plastics produced from algal biomass or by using algal materials. The algal bioplastic is easily biodegradable than compared to commercial plastics. The PHB content present in the algal biomass increases the biodegradable property of plastic thereby decreasing the amount of petroleum used per unit of plastic.

In this research, Algae are used for the production of PHB. Industrial utilization of algae as PHB producers has the advantage of converting waste carbon-dioxide, a greenhouse gas to environment friendly plastics using the energy of sunlight.[3] This research work was focused on the production of biodegradable plastics, polyhydroxybutyrate (PHB) from low cost and easily available raw materials.

MATERIALS AND METHODS

Collection, Isolation And Purification Of PHB Producing Algae In Bold's Basal Agar Medium

Water samples were collected from different freshwater habitats of Tamilnadu. The algae was isolated and purified using microbial techniques primarily with serial dilution which was followed by spread plating and quadrant streaking on Bold's Basal Medium (BBM) agar plates. The purified algal culture was grown in Bold's Basal Medium (BBM).

Screening For PHB Producing Algae

Sudan Black B Staining

The isolate was stained with Sudan Black B stain (Solvent Black 3, HiMedia) It was stained for 10 minutes, washed with water and counter stained with 0.5% safranin for 5 minutes. The slide was observed under the microscope at 1000X magnification.

Nile Blue A Staining Technique

The cells were stained with 1% Nile Blue A for 10 minutes and observed at an excitation wavelength of 460 nm using Fluorescence microscope.

Identification Of PHB Producing Microalgae

Identification Using 18srRNA based molecular method

The PHB producing algae was identified using 18S rDNA sequence. The first ten sequences were selected based on the maximum identity

score and aligned using multiple alignment software program Clustal W. The Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

Extraction of poly – β - hydroxybutyrate

The algal sample was centrifuged for 15 minutes and the pellet was treated with 10 ml of sodium hypochlorite. It was incubated at 30°C for 2 hours. After incubation, it was centrifuged at 5000 rpm for 15 minutes, washed with distilled water and methanol respectively. The supernatant was discarded and the pellet was dissolved in 5 ml of boiling chloroform and the chloroform solution was concentrated to a small volume. A volume of ice cold methanol was added, and refrigerated overnight. The precipitated PHB was collected by centrifugation[6].

Production of PHB Using Inexpensive Organic Substrates

The production of PHB by fermentation, the substrate and recovery costs is expensive which makes their use unattractive. Therefore, the use of inexpensive materials can substantially reduce the substrate cost. The following inexpensive substrates were inoculated with the isolated microalgae for PHB production.

1. Potato boiled water
2. Paddy straw water
3. Rice spent water
4. Rice boiled water.
5. Bengal Black Gram spent water.
6. Molasses
7. Mushroom wastewater and
8. Jack fruit seed powder

The inexpensive substrates were mixed with modified Allen's medium (MAM) in the ratio of 2:1, autoclaved cooled and inoculated with the isolated algal cultures. It was observed for growth and production of PHB.

Preparation of Polymer Blends (PHB - PLA Blends)

Polymer blends were prepared by dissolving PHB in hot chloroform under stirring at 70°C. The PHB-PLA blend films were prepared by conventional solvent casting technique where 3mg of PHB and 1g of Poly lactic acid (PLA) were taken and mixed with hot chloroform and the solvent was slowly evaporated at room temperature.[7]

Determination of Mechanical Properties

- Tensile strength
- Elongation at break ratio.

Polymer films were prepared by solvent casting technique and the polymer films speed of stretching was set at 1.0 mm min^{-1}. Tensile Strength (MPa) and elongation at break (%) was calculated from resulting stress-strain curves.

Characterization of PHB

FTIR Analysis

The polymer extracted from the Rice boiled water was analyzed qualitatively by FTIR to know the presence of different functional groups. Spectra were recorded in 4000cm-1 range.

Analysis of PHB by Scanning Electron Microscope

The size and morphology of the polymer was studied using SEM. The microstructure and surface morphology of PHB was obtained.

NMR Analysis

NMR Analysis was used to determine the quality of PHB structural composition. The proton H1 NMR spectra was obtained at 400 MHz for PHB extracted from rice boiled water.

Differential Scanning calorimeter (DSC) for PHB Powder and PHB Film.

DSC analysis was done using Q 200TA Instruments, Waters, Austria.10-15mg of the extracted polymer was treated to a temperature profile over -30°C to 200°C, at a heating rate of 10°C min-1 for the first heating scan and then held isothermally for 3 minutes. The sample was then cooled to -30°C at 10°Cmin^{-1} by quenching in liquid nitrogen. It was again reheated to 200°C at a heating rate of 10°Cmin^{-1} during a second heating scan.

Thermal Gravimetric Analysis (TGA) for PHB Powder and PHB Film

Thermal stability of the extracted polymer was investigated using Q50 V20.5 Build 30TA Instruments, Waters, Austria, under nitrogen atmosphere. 10-15mg of the extracted polymer was placed in an aluminium pan and then heated from 35 to 700°C with a heating rate of 10°C min^{-1}.

Powder X- Ray Diffraction

To understand the structure of PHB produced by the algae, powder XRD patterns were recorded using an X-ray diffractometer. Data were recorded in 20 ranges of 10°-80 under continuous scan mode using the scan rate of 4° /min.

Applications of PHB Produced

Shelf Life Testing

Shelf life testing was done to determine the durability level of bioplastics as plastic packaging material. In this study, the shelf life test was carried out by placing the bioplastics in a plastic box with limited oxygen or humidity of 45 to 60% to determine the damage caused by microorganisms. The testing process lasted for 90 days. The results were analyzed visually.

Migratory Tests

Overall migration Tests were performed in two liquid food stimulants: ethanol 10% (v/v) and 95% ethanol (v/v) in agreement with the Commission Regulation EU N° 10/2011(Official Journal of European Communities,2011) and isooctane according to the Commission Directive 2002/72/EC (Official Journal of European Communities., 2002). The films were immersed in 25ml of food stimulant in both cases in triplicate.

Biodegradable Products

As PHB can be processed like thermoplastic, it could be used for similar applications as conventional commodity plastics. It can be used to make Biodegradable Spoons, Biodegradable covers, small bowls and pouches.

RESULTS AND DISCUSSION

Collection, Isolation and purification of PHB producing algae in Bold's Basal Agar Medium

The water samples were collected by hand in a plastic bottle from different freshwater habitats of Tamil Nadu which includes Muttukadu lake, Yelagiri lake and Tuticorin pond water. Visible algal colonies were observed after 14 days in Bold's Basal Agar Medium and inoculated into 100 ml Bold's Basal Medium (broth).

Screening, Identification and Extraction of PHB producing Microalgae

The PHB granules appeared as black color granules and cells appeared pink in color (Figure 1). Sudan Black staining is considered as a presumptive test for the detection of PHB. The PHB granules have an affinity for the dye Sudan Black, which is an assumptive test for the presence of PHB. Sudan Black is a fat-soluble dye which has very high affinity for neutral fats and lipids. During staining, the dye leaves the solvent because of its high solubility in lipids than solvent. Therefore, this staining is considered as a presumptive test for the detection of PHB.[8]

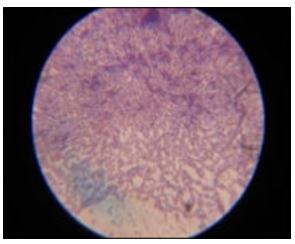

Figure 1 - Sudan Black Staining *C. globosa*

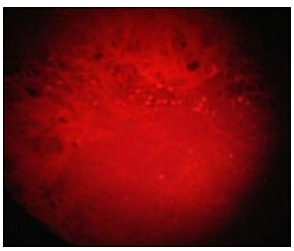

Figure 2 - Nile Blue Staining of *C. globosa*

Nile Blue A is a satisfactory stain for PHB granules and in fact superior to Sudan Black B. It stains more PHB granules than Sudan Black B and is not easily washed from the cell by decolorization. The oxazone form of the dye (Nile Pink) is responsible for the fluorescent staining of PHB.[9] The PHB granules of the isolated algae exhibited a bright orange fluorescence.[10] (Figure 2).

18S rDNA sequence analysis has been used[11] to classify the Chlorophycean algae (Figure 3) and numerous information has helped in the analysis of phylogenetic positions of closely related species.

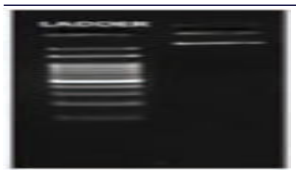

Figure 3 – Electrophoresis for gDNA extraction

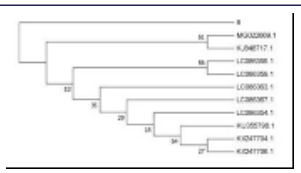

Figure 4 - Molecular Phylogenetic Analysis

Based on 18srRNA sequence, the isolated microalgae was identified as *Chlamydomonas globosa.* Phylogenetic tree was constructed using MEGA 7 (Figure 4).

Fig 5 : PHB extracted from Inexpensive substrates

The PHB granules were extracted and the precipitated PHB was collected by centrifugation (Figure 5).

Production of PHB using Inexpensive Substrates

The readily available cheap substrates can be used as carbon source, which can eventually reduce the high production cost. *Chlamydomonas globosa* effectively utilized Rice boiled water, Potato water and Jack Fruit Seed Powder as an inexpensive substrate for PHB production. *Chlamydomonas globosa* did not utilize other inexpensive substrates like Bengal gram spentwater, mushroom wastewater etc. There was no growth of microalgae when only the inexpensive materials were used as substrates for the production of PHB.

When the inexpensive substrates were mixed with Modified Allen's Medium (selected as it gave good production of PHB) in the ratio of 2:1, maximum growth was observed and PHB was extracted and purified. *Chlamydomonas globosa* uses the medium as the basal substrate for its growth and subsequently uses the organic substrates (for example, Rice Boiled Water) for its further growth thereby enhancing the production of PHB to maximum amount.

Determination of Mechanical Properties and characterization of PHB

The PLA-PHB blend(Figure 6) of 75:25 shows better mechanical performance than PLA confirming that the finely dispersed PHB crystals acts as a filler for PLA matrix.[12]

Fig 6: PHB and PLA polymer blends

FTIR Analysis of PHB Powder and PHB _ PLA Film extracted from Chlamydomonas globosa

The FT-IR analysis of pure PHB, isolated from the strain *Chlamydomonas globosa* revealed that the absorption band occurred at 3436 cm-1 representing the O-H bending as in (Figure 7). The peak at 2929 cm-1 shows the strong -CH2 stretching groups. The medium-strong C=H bond occurred at 2359 cm-1 and 2342 cm-1. The presence of C=O and C-O stretch of ester could be confirmed from the peaks at 1725 cm-1 and from the series of intense peaks located at 1101 cm-1 respectively.[13]

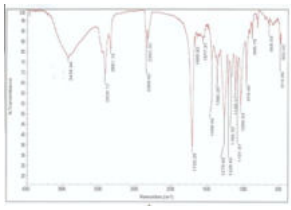

Figure 7 - FTIR Analysis of PHB Powder

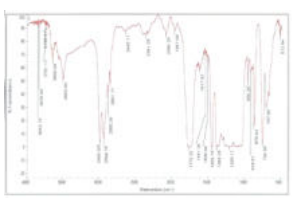

Figure 8 - FTIR Analysis of PHB _ PLA Film

FTIR spectra display the absorption bands of PLA based systems. Several absorption bands specific for PHB and PLA were detected (Figure 8). The differences in initial crystallinity of polymers must be considered (PLA – amorphous; PHB - Crystalline). The ν(c=o) band widths for PLA and PHB differed. The FTIR spectra of PLA_PHB blends shows two major carbonyl stretching bands which is due to PLA and PHB.[14]

Analysis of PHB by Scanning Electron Microscope (SEM)

The microstructure and surface morphology of PHB was obtained using Scanning electron microscopy. It shows microstructures that are porous and interconnected and has a strong tendency to form multigrain agglomerates (Figure 9).

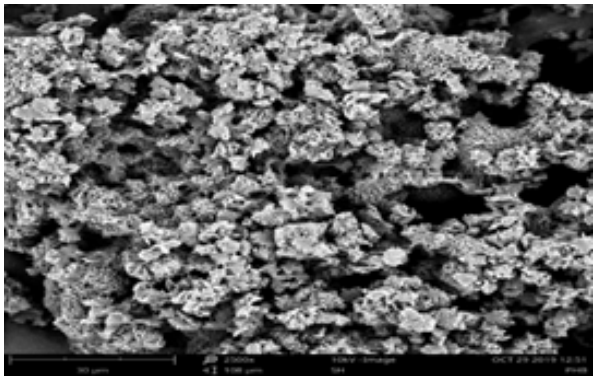

Figure 9 - Analysis of PHB by Scanning Electron Microscope (SEM)

NMR Analysis of PHB Powder extracted from Chlamydomonas globosa

The HNMR showed a predominant peak at 1.254 ppm which corresponded to the terminal methyl (-CH_3) group of the hydroxybutyrate (HB molecule). The peaks from 2.628 to 2.451 correspond to the (-CH_2) methyl protons and the -CH proton of the PHB molecule corresponded to the 5.289 peak of the 1HNMR spectrum (Figure 10).

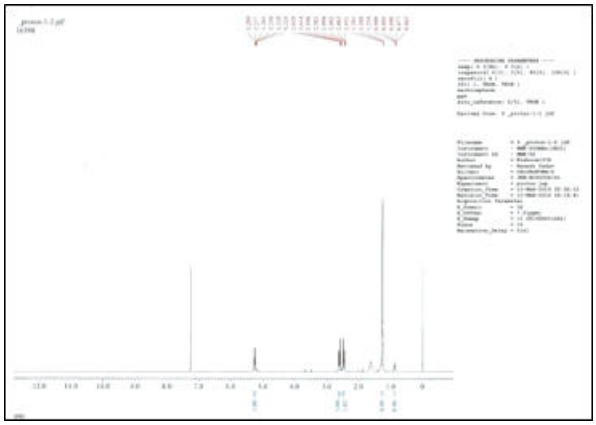

Figure 10 - NMR Analysis of PHB Powder extracted from *Chlamydomonas globosa*

Powder XRD Analysis of Chlamydomonas globosa

The XRD study was carried out to check crystalline structure of PHB. The XRD diffractogram shows two prominent peaks at 15.71° and 18.34°. The presence of intense peak at 15.71° indicates the crystalline nature of the polymer (Figure 11). The diffractogram is almost identical with that by [15] for the standard PHB and also for the PHB produced by *Cuprivavidus necator* by solid-state fermentation.

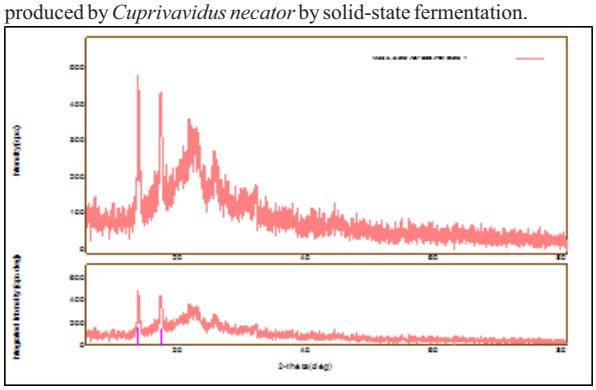

Figure 11 - Powder XRD Analysis of *Chlamydomonas globosa*

DSC Thermogram of Powdered PHB and PHB+PLA Film

From the DSC thermogram (Figure 12), the degree of crystallinity (%) for *Chlamydomonas globosa* was found to be less which clearly explains that they have good mechanical properties.[16]

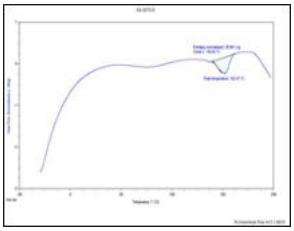

Figure 12 - DSC of Powdered PHB

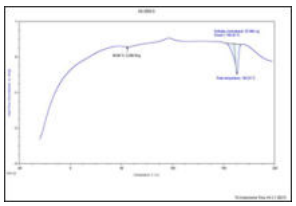

Figure 13 - DSC for PHB + PLA Film

The DSC results for pure PHB (35%) showed higher crystalline degree in comparison to PHB – PLA Blends (Figure 13). The crystallinity of PHB in the blends were maintained between 17% and 19%. The melting points of the PHB and PLA film ranged between 160°C and 140°C.[17]

TGA for Powdered PHB and TG-DSC for PHB+PLA

The thermal degradation at maximum decomposition temperature is associated with the ester cleavage of PHB component by β – elimination reaction.[18] reported that the PHB was degraded in two stages and completely degraded at 300° C (Figure 14).

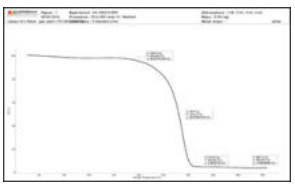

Figure 14 - DSC for PHB + PLA Film

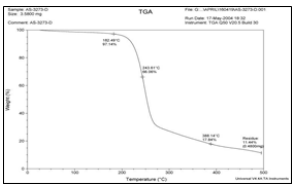

Figure 15 - TGA for Powdered PHB

The thermal decomposition patterns of PHB-PLA blend follow different reaction for PHB. Maximum decomposition temperature also increases from 275°C - 500°C (Figure 15).

Applications of PHB Produced

Shelf Life Testing and Migratory Tests

The shelf life of bioplastics was found to be good. The bioplastics were observed visually and there was no change in the surface morphology of the bioplastics which indicates that PHB-PLA films can be used as an effective food packaging material. According to (Bucci & Tavares, PHB packaging for the storage of food products, 2005), the challenges faced by the food packaging industry is its effort to find out the durability of the packaging with product shelf life. The packaging material must remain stable without changes in mechanical or barrier properties and also should be stable during storage until disposal.

The main criteria necessary for the biopolymeric material to be used in food packaging applications is their overall migration limit, which should be lower than those limits established in the current legislation (European Commission Regulation, 2011) [19] ensures that the total amount of non-volatile substances that might transfer into foodstuff from polymers will not cause a risk to the consumer.

The overall migration values of PLA-PHB composites in ethanol 10% (v/v) aqueous solution; 95% ethanol (v/v) aqueous solution and Isooctane were determined. It was observed that in all cases, the overall migration values were not detected. There was no increase in the migratory properties of PHB-PLA blends.

Biodegradable Products

As PHB can be processed like thermoplastic they can be used for the production of small covers and pouches (Figure 16) biodegradable spoons (Figure 17) and small bowls.

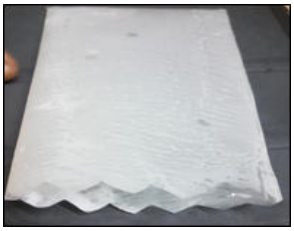

Fig 16: Pouch made with (PHB & PLA)

Fig 17: Biodegradable Spoons

Fig 18: Biodegradable Heart Shaped Coloured Bowl (with Turmeric)

Natural colors like yellow from turmeric powder were added to the polymer blends (Figure 18). The addition of turmeric to the blend increases the shelf life of the product as it has potent antibacterial, antifungal and antiviral properties.

CONCLUSION

Rising concern over damaging effects of petroleum-based plastics on environment has led to this research which is based on the production of biodegradable plastic- polyhydroxybutyrate from inexpensive substrates. Although there is more work done by using bacteria for the production of PHB using inexpensive substrates, not much work has been reported **using microalgae** for the production of PHB, using these inexpensive substrates like Rice Boiled Water, Potato Boiled Water, Paddy Straw, Jackfruit Seed Powder etc.

From the above study, we can conclude that the isolated freshwater microalgae can be used to produce the biodegradable polymer called PHB. This work also covers the identification, characterization, biosynthesis, quantification and application in different fields.

PHB's success in market can be greatly encouraged with new governmental legislation. The Tamil Nadu government has banned the manufacture, sales, storage and usage of items such as plastic paper, cups, water sachets, straws and carries bags since January 2019. If moves like these continue, there's a better chance that will see PHB in our supermarkets.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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