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BIOPLASTICS [POLY-ß-HYDROXYBUTYRATE(PHB)] FROM *PLEODORINA STARRII*

Biotechnology

Rebecca Robert PG & Research Department of Biotechnology, Women's Christian College, Chennai, India.

Priya R. Iyer* PG & Research Department of Biotechnology, Women's Christian College, Chennai, India. *Corresponding Author

ABSTRACT

Our reliance on petroleum derived plastic materials and their uncontrolled utilization are the major cause of waste accumulation on landfill and release of greenhouse gases. A biologically synthesized plastic material namely Poly-ß- hydroxybutyrate (PHB) has gorgeous physical and mechanical properties similar to conventional plastics produced from petroleum products.PHB are completely biodegradable and leave behind no residue. Algae are used to produce PHB, for bioplastic production which offers an opportunity in economic efficiency by reduced costs. Pleodorina starrii was isolated from different freshwater sources and screened for PHB production using Sudan black B and Nile Blue Stain. The PHB was extracted using hot chloroform and the amount of PHB produced was estimated by reading the absorbance at 235nm.Characterization of extracted PHB was carried out by FTIR and NMR. The extracted PHB was blended with other polymers to improve its physical characteristics. The thermal properties of Polymer was studied using Differential Scanning calorimetry(DSC), Thermogravimetric Analysis(TGA), Powder X-ray Diffractometer(XRD). Further the polymer showed good tensile strength with a low extension to break ratio comparable to petrochemical plastic. Applications of PHB include Shelf Life Testing, Migratory Tests and production of various biodegradable products like small covers and pouches.

KEYWORDS

Biodegradable-Biopolymers-Inexpensive- Nile Blue- Petrochemical-PHB-Shelf Life-Tensile strength.

INTRODUCTION

The history of plastic begins from 1862 by Alexander Parkes. Biodegradable form of plastic was first characterized in the mid-1920s by French researchers. This molecule is called Polyhydroxybutyrate (PHB). Many different types of bacteria and algae produce it as food storage material[1]. Biodegradable plastics can decompose into carbon-dioxide, methane, water, inorganic compounds or biomass via microbial assimilation. PHB has many different features such as thermoplastic process ability, non-toxicity and high crystalline features. PHB is a water-resistant polymer and completely biodegradable.

Algae serve as an excellent feedstock for bioplastic production owing to its many advantages such as high yield and the ability to grow in a range of environments[2]. The algal bioplastic are easily biodegradable than compared to commercial plastics. In this paper, microalgae 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.

MATERIALS AND METHODS

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

Samples were collected from different freshwater habitats of Tamilnadu .The algae was isolated and purified using basic microbial techniques primarily with serial dilution 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, Identification and Extraction of PHB producing Microalgae

The isolate was stained with Sudan Black B stain and Nile Blue Stain.The PHB producing algae was identified using 18S rDNA based molecular method. The PHB was extracted using Hot Chloroform Method.

Production of PHB Using Inexpensive Organic Substrates

Various inexpensive substrates were inoculated with the isolated microalgae for PHB production. They include Potato boiled water, rice boiled water, jack fruit seed powder, paddy straw water etc.

Preparation of Polymer Blends (PHB-PLA Blends)

Polymer blends were prepared with PHB by conventional solvent casting technique[3].

Mechanical Properties Determination and characterization of PHB

The polymer films were cut into a rectangular shape and the speed of stretching was set at 1.0 mm min^{-1}. Tensile strength (MPa) and elongation at break (%) were calculated from resulting stress-strain curves. The extracted PHB polymer and the powder were characterized using various analytical techniques like FTIR, NMR, Scanning electron microscope, DSC, TGA and powder XRD.

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

Samples were collected 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 were observed as black color granules and cells appeared pink in color. Sudan Black staining is considered as a presumptive test for the detection of PHB[4](Figure 1).

Fig 1 : Sudan Black Staining

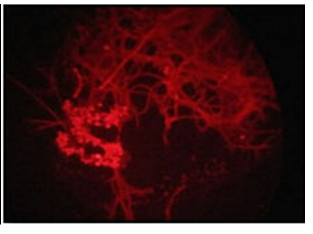

Fig 2 : Nile Blue Staining

The isolated algae exhibited a bright orange fluorescence for PHB accumulation by Nile blue staining method5 (Figure 2). The isolated algae producing PHB was observed microscopically (Figure 3).

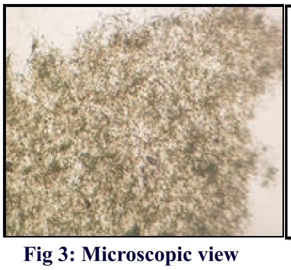

Fig 3: Microscopic view of Pleodorina starrii

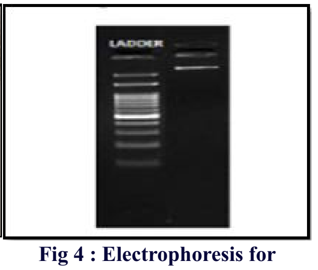

Fig 4 : Electrophoresis for gDNA extraction

Based on 18S rRNA sequence (Figure 4), the isolated microalgae was identified as *Pleodorina starrii.*

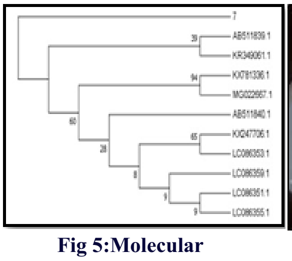

Fig 5:Molecular Phylogenetic Analysis

Fig 6:PHB extracted from Inexpensive substrates

Phylogenetic tree was constructed using MEGA 7(Figure 5).The PHB granules were extracted and the precipitated PHB was collected by centrifugation (Figure 6).

Production of PHB using Inexpensive Substrates

The use of readily available cheap substrates as carbon source can reduce the high production cost. Although there is more work done by using bacteria for the production of PHB using inexpensive substrate, not much work has been reported using microalgae for the production of PHB. Inexpensive substrates like Rice Boiled Water (Figure 7), Potato Boiled Water, Paddy Straw, Jackfruit Seed Powder etc. are effectively used by Pleodorina starrii as an inexpensive substrate for PHB production.

Fig 7: Growth of P. *starri* in Rice Boiled Water

Mechanical Properties Determination and characterization of PHB

Fig 8: PHB and PLA polymer blends

The PLA-PHB 75:25 blend shows better mechanical performance than PLA confirming that the finely dispersed PHB crystals acts as a filler for PLA matrix[6](Figure 8).

FTIR Analysis of PHB Powder and PHB _ PLA Film extracted from *Pleodorina starrii*

The FT-IR analysis of pure PHB, isolated from the strain *Pleodorina starrii* (Figure 9) revealed that the absorption band occurred at 3436cm-1 representing the O-H bending. The peak at 2929cm-1 shows the strong -CH2 stretching groups. The medium- strong C=H bond occurred at 2359cm-1 and 2342cm-1. The presence of C=O and C-O stretch of ester could be confirmed from the peaks at 1725cm-1 and from the series of intense peaks located at 1101cm-1 respectively[7].

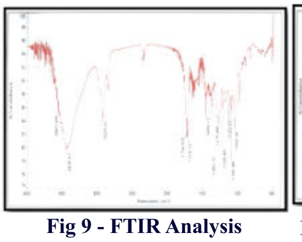

Fig 9 - FTIR Analysis of PHB Powder

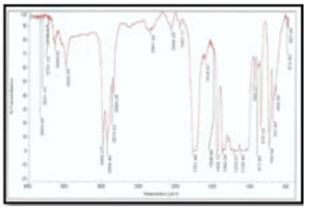

Fig 10 - FTIR Analysis of PHB _ PLA Film

FTIR spectra display the absorption bands of PLA based systems (Figure 10). Several absorption bands specific for PHB and PLA were detected. 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[8].

Analysis of PHB by Scanning Electron Microscope (SEM)

The microstructure and surface morphology of PHB was obtained using Scanning electron microscopy (Figure 11).

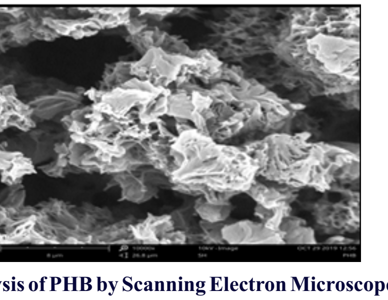

Fig 11 - Analysis of PHB by Scanning Electron Microscope (SEM)

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It shows microstructures that are porous and interconnected and has a strong tendency to form multigrain agglomerates.

NMR Analysis of PHB Powder extracted from *Pleodorina starrii*

The HNMR showed a predominant peak at 1.254ppm which corresponded to the terminal methyl($-CH_3$) group of the hydroxybutyrate (HB molecule).

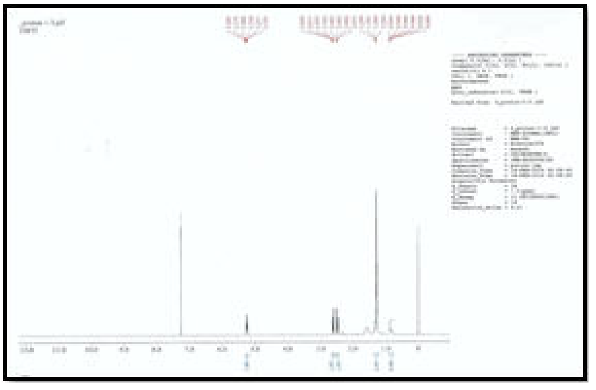

Fig 12 - NMR Analysis of PHB Powder extracted from *Pleodornia starrii*

The peaks from 2.628 to 2.451 correspond to the (-CH2) methyl protons and the -CH proton of the PHB molecule corresponded to the 5.289 peak of the 1HNMR spectrum (Figure 12).

Powder XRD Analysis of *Pleodorina starrii*

The XRD study was carried out to check crystalline structure of PHB (Figure 13). 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[9].

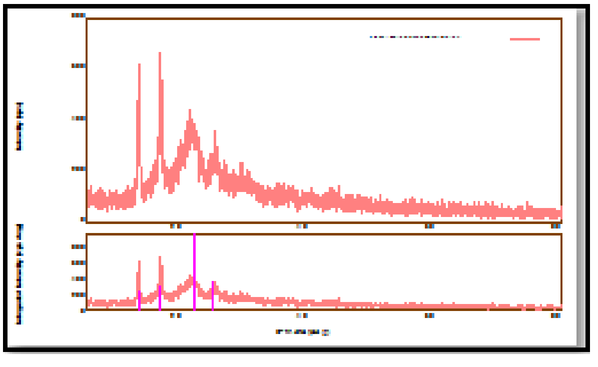

Fig 13 - Powder XRD Analysis of *Pleodornia starrii*

DSC Thermogram of Powdered PHB and PHB+PLA Film

From the DSC thermogram (Figure 14), the degree of crystallinity (%) for *Pleodorina starrii* was found to be less, which clearly explains that they have good mechanical properties[10].

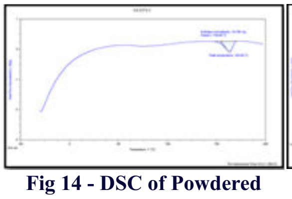

Fig 14 - DSC of Powdered PHB

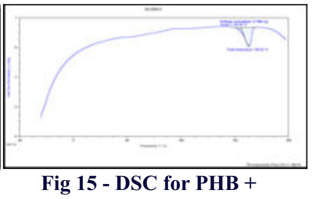

Fig 15 - DSC for PHB + PLA Film

The DSC results for pure PHB (35%) showed higher crystalline degree when compared to PHB – PLA Blends. The crystalline degree of PHB in the blends was maintained between 17% and 19%. The melting points of the PHB and PLA film ranged between 160°C and 140°C11 (Figure 15).

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 [12]reported that the PHB was degraded in two stages and completely degraded at 300° C (Figure 16).

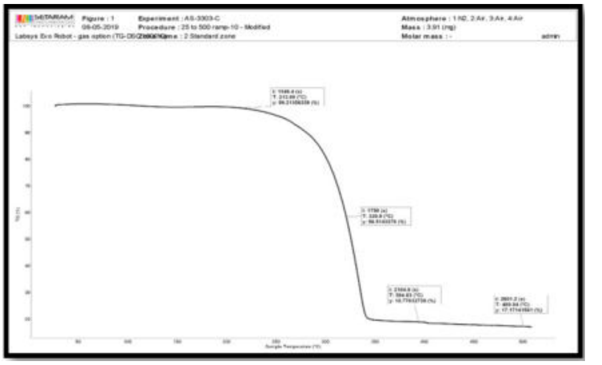

Fig 16 - DSC for PHB + PLA Film

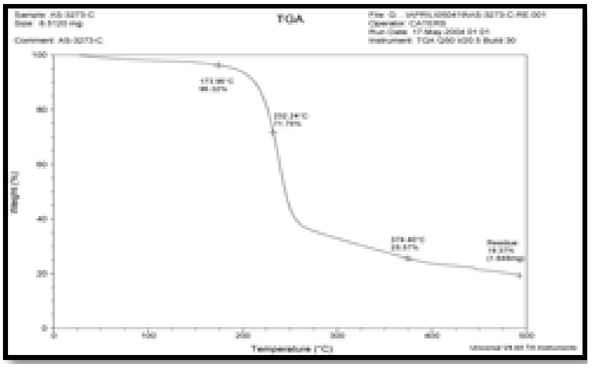

Figure 17 - TGA for Powdered PHB

The thermal decomposition patterns of blends follow different reaction for PHB. Maximum decomposition temperature also increases from 275°C - 500°C (Figure 17).

Applications of PHB Produced

Shelf Life Testing and Migratory Tests

The shelf life of bioplastics was found to be good (Figure 18). 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 (Figure 19).

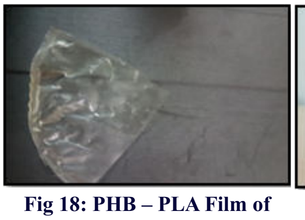

Fig 18: PHB – PLA Film of *Pleodorina starrii*

Fig 19: Shelf life Testing

The overall migration values of PLA-PHB composites in ethanol 10% (v/v) aqueous solution; 95% ethanol (v/v) aqueous solution and Isooctane were determined13.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 biodegradable spoons, small bowls, small covers and pouches.

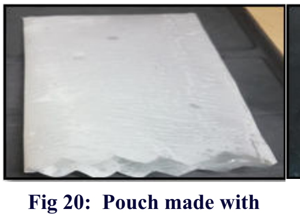

Fig 20: Pouch made with biodegradable polymer

Fig 21: Small covers for food packaging

Natural colors like yellow from turmeric powder were added to the polymer blends. The addition of turmeric to the blend increases the shelf life of the product as it has potent antibacterial, antifungal and antiviral properties (Figures 20 and 21).

CONCLUSION

Pleodornia starrii was isolated from freshwater sources. This microalgae has the potential to produce biopolymers like PHB from CO_2 as the carbon source, and the yield of PHB can be increased by various nutrient limiting conditions. This technology has shown a way, for the production of the algae-based bioplastics which are still under the research phase and are far from commercialization.

A major problem for extensive production and commercialization of PHB is its high production cost as compared with plastics derived from petrochemicals. The alternative is to choose economically feasible and which are readily available carbon substrates for both growth and for efficient PHB production. Use of inexpensive substrates like rice boiled water, potato boiled water etc. serves dual purposes of reducing the cost of biodegradable plastics and also by reducing the environmental pollution caused by these conventional plastics.

Acknowledgement

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Conflict of interest

The authors have no conflicts of interest to declare.

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