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Research Article

ISOLATION AND OPTIMIZATION OF PHB (POLY- B- HYDROXYBUTYRATE) FROM *Pseudoneochloris marina*

Rebecca Robert and Priya Iyer

Department of Biotechnology,Women's Christian College, Chennai

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ABSTRACT

Poly-β-hydroxybutyrate (PHB) can be used as an effective thermoplastic and has many characteristics similar to those of standard commercial plastics like polypropylene. PHB based plastic substitutes are less flexible than traditional plastics; they 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. Pseudoneochloris marina was isolated from different freshwater sources and screened for PHB production using Sudan black B and Nile Blue Stain. The production of PHB was optimized using different media and under various parameters like Aeration; Effect of phosphate and Sodium acetate etc. 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.

INTRODUCTION

Bioplastics or organic plastics are a form of plastics derived from renewable biomass sources such as vegetable oil, corn, starch, pea starch unlike fossil-fuel plastics derived from petroleum. 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 (D.B.Falcone, 2004). Biodegradable plastics can decompose into carbon-dioxide, methane, water, inorganic compounds or biomass via microbial assimilation. Algae serve as an excellent feedstock for plastic production owing to its many advantages such as high yield and the ability to grow in a range of environments. (Greg Stevens, 2010). PHB has many different features such as thermoplastic process ability, non-toxicity and high crystalline features. This suggests that PHB could be a good alternative to fossil plastics. The melting point is about 179°C and the polymer is not branched. Thus, it can be melted easily during production. PHB is a water-resistant polymer and completely biodegradable. "BUGS MAKE PLASTIC" ran the headline when PHB was first commercialized in the early eighties. Most plastics are made from oil but this one is made naturally, all the time, by microorganisms and supply is limitless. When extracted, this PHB solidifies into a polymer very similar to traditional plastics like polythene. In this paper, 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 environmentally friendly plastics using the energy of sunlight.

MATERIALS AND METHODS

Sample Collection

Samples were collected from different freshwater habitats of Tamilnadu which includes Muttukaddu lake, Yelagiri lake, Tuticorin; Panderavellai; Alandur and WCC pond water.

Isolation and Purification of PHB producing Algae

The algal species were isolated and purified from different sources using basic microbial techniques primarily with serial dilution and followed by spread plating and quadrant streaking on BBM (Bold's Basal Medium) agar plates. Morphological identification was performed through microscopic observations.

Identification using 18S RDNA Based Molecular Method

The algal species were identified using 18S RDNA sequence. Based on maximum identity score, first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

Corresponding author:* **Priya Iyer
Department of Biotechnology,Women's Christian College, Chennai

Culture Conditions

The purified axenic cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL of BBM. Experimental cultures were incubated at 25 $\pm$ 2°C, 14/10 light/dark cycles with illumination of 3000 lux under cool white fluorescent lamps. Every day the cultures were mildly shaken by hand for 10 minutes.

Screening for the Production of PHB Using Sudan Black Staining Technique

The isolates were stained with Sudan Black stain. The samples were stained for 10 minutes with Sudan Black Solution, rinsed with water and counter stained with 0.5% safranin for 5 minutes. The slide was observed under the microscope at 1000x magnification.

Nile Blue Staining Technique

Heat fixed cells were treated with 1% Nile blue for 10 minutes and was observed at an excitation wavelength of 460 nm.

Optimization Studies

Optimization of Media

The following media were prepared, and the isolated algal culture was inoculated and incubated at 14/10 light/dark cycle with illumination of 3000 lux under cool white fluorescent lamps. Every day the culture was mildly shaken by hand for 10 minutes.

Media Used

- Cyanophycean medium
- Chu 10 Medium
- Fogg's Nitrogen free Medium
- BG 11 Medium
- Chu 10 Modified Medium (CMM)
- Algae culture Medium
- Modified Allen's Medium (MAM)
- Hughes and Gorham Medium
- Bold's Basal Medium (BBM).

Effect of Aeration on PHB production

Pseudoneochloris marina was inoculated into three different selected media. Based upon their growth and production of PHB these media were selected. They include Bold's Basal Medium (BBM); Modified Allen's Medium (MAM) and Chu 10 modified medium (CMM). Air was supplied into the medium through the aerator. After two weeks, growth was observed and PHB was extracted. The amount of PHB extracted was quantified using UV spectrophotometer at 235nm.

Effect of Phosphate Deficiency on PHB Production

To study the impact of phosphate deficiency on PHB accumulation, *Pseudoneochloris marina* was directly grown in all the 3 media which were devoid of phosphates.

Effect of Darkness on PHB Production

To study the impact of darkness on PHB accumulation, *Pseudoneochloris marina* was directly grown in Bold's Basal Medium (BBM) , Modified Allen's Medium (MAM) and Chu 10 modified medium (CMM) and incubated in darkness.

Effect of sodium acetate on PHB production

1g/L of sodium acetate was added to Bold's Basal Medium (BBM), Modified Allen's Medium (MAM) and Chu 10 modified medium (CMM). The culture was incubated, observed for growth and PHB was extracted.

Effect of pH on PHB production

For pH optimization, the selected algal culture was inoculated into Bold's Basal Medium (BBM), Modified Allen's Medium (MAM) and Chu 10 modified medium (CMM) of various pH ranging from pH2, pH4, pH6, pH8 and pH10. The culture was incubated, and the optimum pH was determined based on the amount of PHB produced.

Effect of Nitrogen on PHB production

The Effect of Nitrogen Deficiency on the Production of PHB was Determined under the Following Criteria

1. Media for control treatment (with nitrogen)
2. Media with 50% nitrogen deficiency
3. Media with 100% nitrogen deficiency.

The cultures were incubated, and the effect of nitrogen deficiency was determined based on the amount of PHB produced.

Effect of Nitrogen and Phosphate Deficiency

The effect of nitrogen and phosphate deficiency was determined under the following criteria for *Pseudoneochloris marina*.

1. Media without nitrate and without phosphate.
2. Media with ½ nitrogen and ½ phosphate.

Determination of Standard Curve

According to Khanafari *et al*., 2006 the standard curve was derived by preparing the PHB standard solution at different concentration (10-100µg/ml). About 2 ml of concentrated sulphuric acid was added to all the tubes and kept in boiling water bath for 10 minutes for the conversion of PHB into crotonic acid. After cooling, the absorbance was measured at 235 nm using UV spectrophotometer and standard graph was plotted. About 2 ml of concentrated sulphuric acid was used as blank. Similar procedure was carried out for all samples. The readings were plotted in standard graph of crotonic acid and concentrations of PHB in the sample were determined.

Extraction of Poly- β- Hydroxybutyrate

100 ml of sample was taken and centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded, and the pellet was treated with 10 ml of sodium hypochlorite and the mixture was incubated at 30°C for 2 hours. After incubation, the mixture was centrifuged at 5000 rpm for 15 minutes and then washed with distilled water and methanol respectively.

After washing, the pellet was dissolved in 5 ml of boiling chloroform. The chloroform solution was concentrated to a small volume. A volume of cold methanol was added, and the sample was refrigerated overnight. The precipitated PHB was collected by centrifugation.

Purification of Poly- β- hydroxybutyrate

Extracted PHB crystals were re-dissolved in 5 mg in 5 ml of chloroform in a test tube in waterbath at 100°C for 20 minutes and filtered through Whatman no: 1 filter paper and chloroform were evaporated by pouring the solution on a sterile plate and kept at 4°C in a fridge. After some time, powder was collected from Petri dishes by scratching.

Production of PHB using Inexpensive Substrates

The PHB production by fermentation, the substrate and recovery costs are high, making their use unattractive. Therefore, the use of waste materials can substantially reduce the substrate cost. The following inexpensive substrates were used.

1. Potato boiled water
2. Hay water
3. Rice spent water
4. Rice boiled water.
5. Bengal black gram spent water.
6. Molasses
7. Mushroom waste.
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 *Pseudoneochloris marina*. It was observed for growth and PHB production.

Characterization of PHB

FTIR Analysis

The polymer extracted from the fermentation medium (Rice boiled water) was analyzed qualitatively by FTIR to know the presence of different functional groups. 1 mg of PHB was dissolved in 5 ml of chloroform. The chloroform was evaporated and KBr pellet was prepared with the resulting PHB. Spectra were recorded in 4000cm-1 range.

NMR Analysis

NMR Analysis was used to determine the quality of PHB structural composition. The proton H1 NMR was done for PHB extracted from rice boiled water.

RESULTS

Isolation and Identification of PHB producing Algae

Based on microscopic observations and 18S RDNA sequence the isolate was identified as *Pseudoneochloris marina.*

Screening for the Production of PHB using Sudan Black and Nile Blue Staining Technique

The PHB granules were also recognized by their affinity for the dye Sudan Black, which is a presumptive test for the presence of PHB. The stained preparations were examined under a compound microscope with an oil immersion lens for determining cellular PHB accumulation. The PHB granules were observed in black color and cells were pink in color.

Pseudoneochloris marina showed positive results for PHB accumulation through Nile blue staining method. The oxazone form is probably formed from the basic oxazine dye Nile Blue A by spontaneous oxidation in aqueous solution thereby producing fluorescence (Ostle & Holt, 1982). PHB inclusions exhibited a bright orange strong fluorescence.

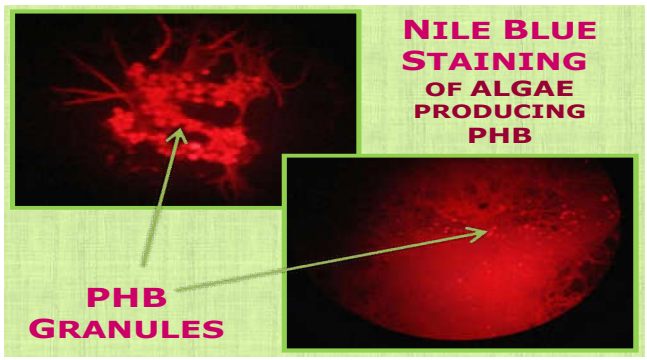

Fig 1 Nile Blue staining of Algae producing PHB

Optimization Studies

Optimization of media

Growth was observed after 2 weeks. There was no growth in Fogg's Nitrogen Free Medium. BBM; Modified Allen's Medium and Chu 10 modified medium showed more amount of PHB production than others. These 3 Medias were further used for PHB production. So far, in the studies made on PHB production, it was observed that BG 11 medium was used but studies have to be developed for more productive results that can contribute to production. As observed in this study, in both biomass and PHB production, Modified Allen's Medium, BBM and CHU 10 modified medium were found to be more suitable for PHB production (Yavuez *et al.,* 2006).

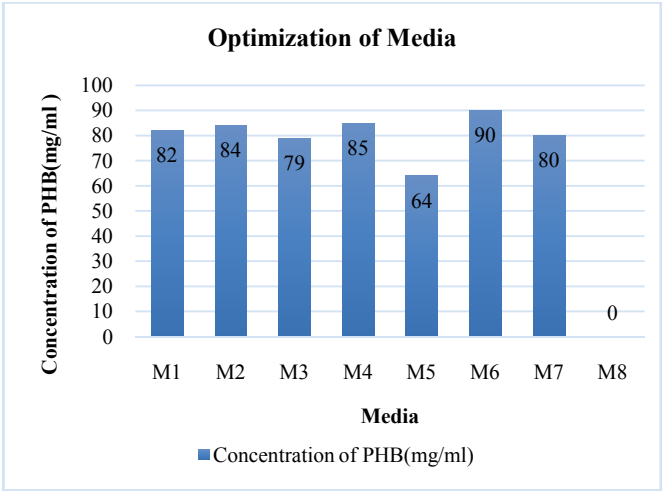

Fig 2 Optimization of Media

*M1 - Cyanophycean Medium; M2 - Chu 10 Medium; M3 - BG11 Medium; M4 - Chu 10 modified medium; M5 - Algae culture Medium; M6 - Modified Allen's Medium; M7 - Bold's Basal Medium; M8 - Hughes & Gorham Medium.

Effect of Aeration on PHB Production

After 2 weeks, growth was observed and PHB was extracted. Aeration conditions have an impact on PHB accumulation. Cell growth increased at higher aeration rates and maximum biomass production was observed (Alejandra *et al.,* 2010).

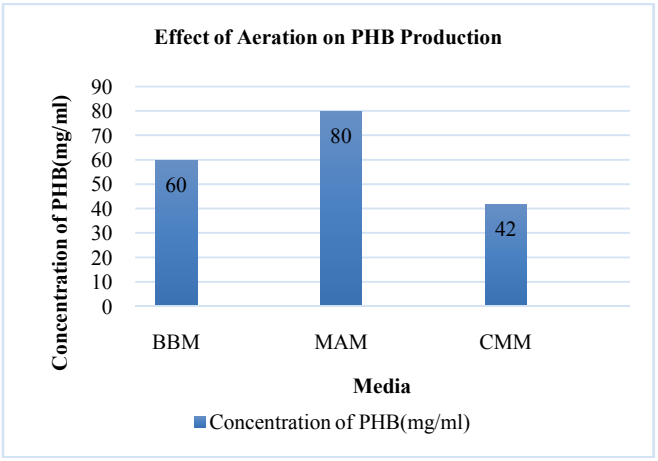

Fig 3 Effect of Aeration

Effect of Phosphate Deficiency on PHB Production

Growth was observed within 7 days of cultivation in Phosphate starved medium. Limitations of phosphorous appeared to be a suitable stimulant for PHB production (Lee *et al.,* 1994)

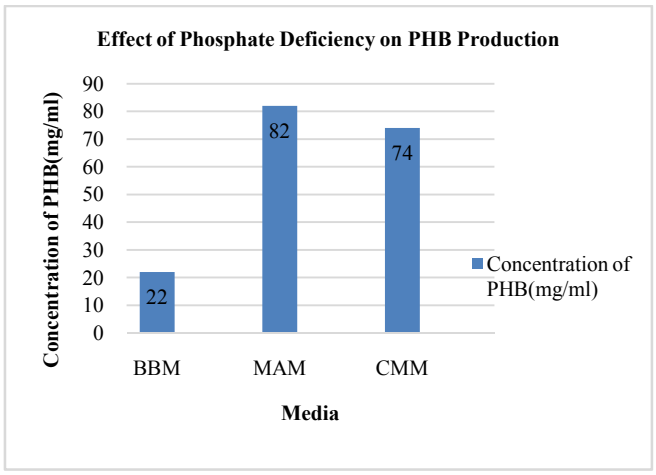

Fig 4 Effect of Phosphate deficiency

Effect of Darkness on PHB Production

There was no production of PHB in darkness, which clearly explains that algae have the ability to accumulate PHB under photoautotrophic condition (Byrom, 1987).

Effect of Sodium acetate on PHB Production

Growth occurred within 4 days of incubation, which clearly shows that sodium acetate activates the growth as well as it increases the production of PHB. Normally the PHB production of the organism was induced by α-ketothiolase enzyme. The addition of sodium acetate to medium will monitor the acetyl CoA activity. Hence the medium was optimized by using sodium acetate (Uma Maheswari and Ahilandeswari, 2011).

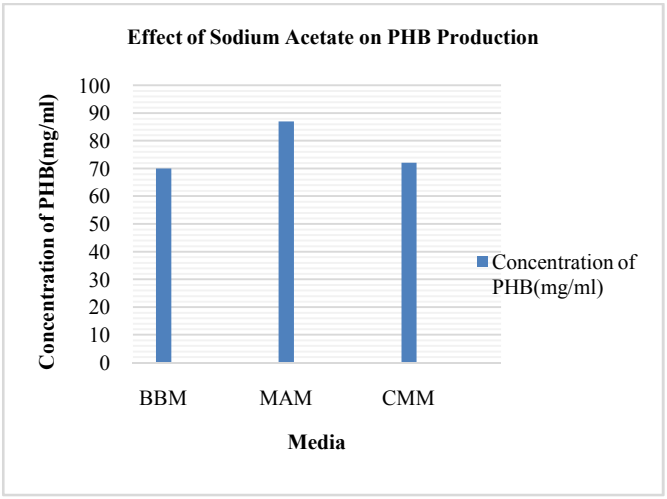

Fig 5 Effect of sodium acetate

Effect of Nitrogen on PHB production

Growth was observed within 4 days of cultivation in media with nitrogen.

In media with 50% nitrogen deficiency, growth was observed only in Bold's Basal Medium. In media with 100% nitrogen deficiency, no growth was observed in any of the medium. Nitrogen deficiency did not act as a suitable stimulant for PHB production (Kanokphorn *et al*., 2008)

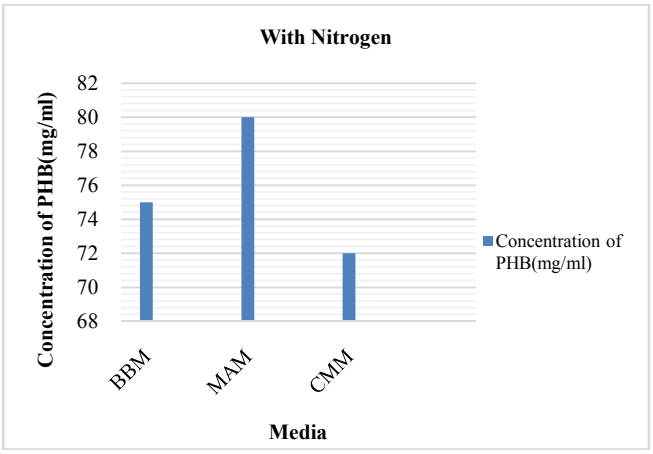

Fig 6 Media with nitrogen

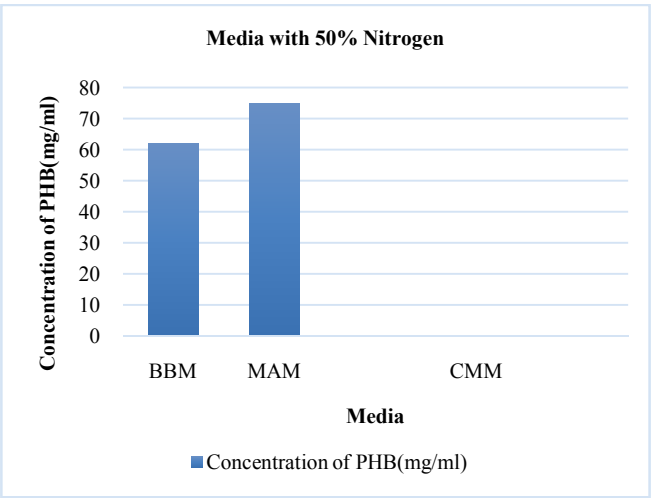

Fig 7 50% Nitrogen Deficiency

Effect of pH on PHB Production

A pH variation from acidic to alkaline was carried out for the production of PHB. Different pH was maintained in the medium and its effect on PHB production was evaluated. The results were tabulated. There was no growth at pH 2 and 4 for BBM and Modified Allen's Medium. Chu 10 Modified Medium showed no growth at pH 2, 4 and 6 also. Acidic pH was not suitable for PHB production (Panda *et al.,* 2006), so also the high alkaline pH is essential (Sundaramoorthy Balaji *et al.,* 2012).

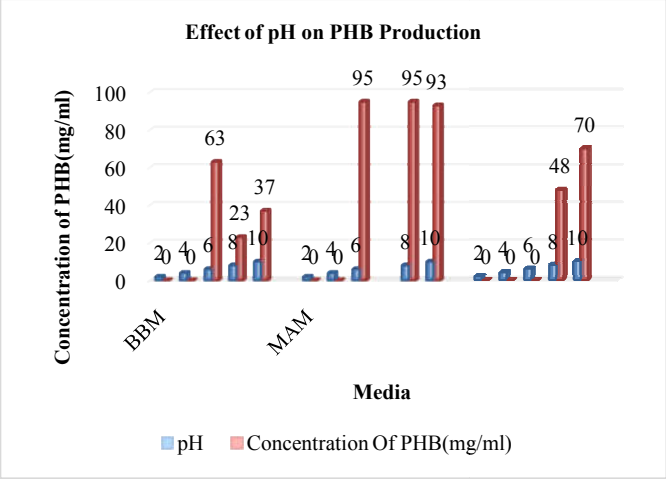

Fig 8 Effect of pH

Effect of Nitrogen and Phosphate Deficiency

1. Media without nitrate and phosphate
   No growth was observed.
2. Media with ½ Nitrogen and ½ phosphate
   No growth was observed.

Although many papers (Onder Uysal *et al.,* 2016) reports the accumulation of PHB under nitrogen and phosphate deficient conditions, this algae *Pseudoneochloris marina* was not able to grow when there is a deficiency in nitrogen and phosphorus.

Production of PHB using Inexpensive Substrates

Rice boiled water was mixed with MAM in the ratio of 2:1 autoclaved cooled and inoculated with *Pseudoneochloris marina*. There was increase in biomass and PHB production. Although many other inexpensive substrates were used, rice boiled water was found to be the most effective inexpensive substrate (Mahitha *et al.,* 2016).

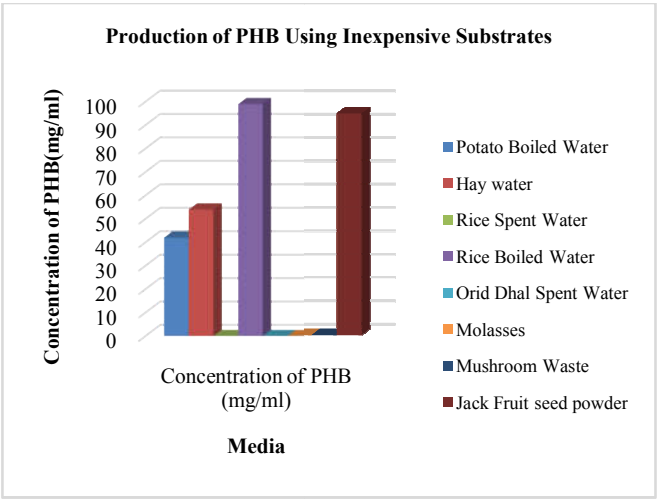

Fig 9 Production of PHB using Inexpensive Substrates.

Characterization of PHB

FTIR Analysis

The FT-IR analysis of pure PHB isolated from the strain *Pseudoneochloris marina* revealed that the absorption band occurred at 3466 cm^{-1} representing the O-H bending. Medium-strong C=H bond occurred at 2359 cm^{-1}. A medium-strong CH3 stretching bond occurred at 1545 cm^{-1}.A medium strong C-H bond occurred at 1466 cm^{-1}.A medium weak C-O stretching bond occurred at 1116cm^{-1}. Misra *et al.,* 2000 suggested that the IR spectra of the intact cells with positive absorption at 1680 cm^{-1} to 1724 cm^{-1} could be used as a tool to screen PHB producing organisms.

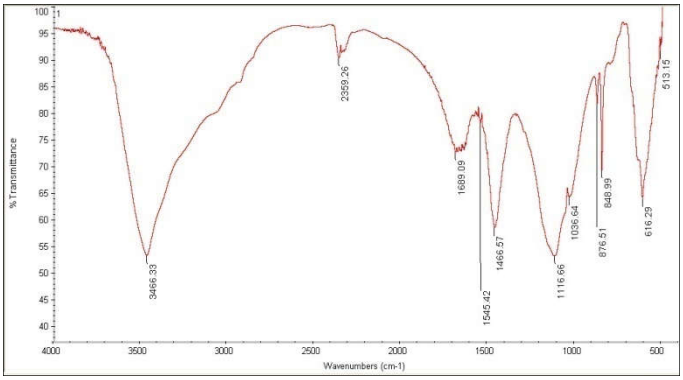

Fig 10 FTIR Analysis

NMR Analysis

NMR analysis was used to determine quality of PHB structural composition. The 1HNMR spectra obtained from PHB sample produced from *Pseudoneochloris marina* demonstrated the presence of 3.2 and 1 proton at chemical shifts 1.2, 2.4-2.6 and 5.3 respectively representing CH_3, CH_2 and CH groups. The molecular composition of the polymer as indicated by chemical shifts, generates a structure of (CH_2-CH) backbone and assigned the presence of (CH_3) group. Yu & Marchessault, 2000 identified CH3 at 1.25 ppm, CH2 at 2.42 ppm. Pal & Paul, 2002 identified the chemical shifts 1.2, 2.4-2.6 & 5.3 representing the CH_3 and CH_2 and CH groups. The results of the present study, exactly matched with above mentioned studies, which confirmed the product as PHB.

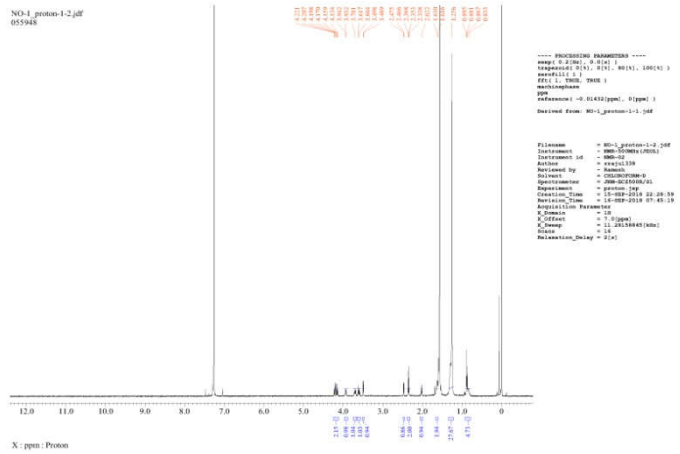

Fig 11 NMR analysis

DISCUSSION

In this study, *Pseudoneochloris marina has* been isolated from different freshwater sources. PHB is accumulated under conditions of nutrient deprivation or in the presence of excess acetate. Three different media were optimized for the production of PHB.

Aeration is an important criterion for the production of PHB in algae. Cell growth increased at higher aeration rates, and maximum biomass production was also observed. Maximum production was observed in Modified Allen's Medium. Phosphorous starved media appeared to be a suitable stimulant for PHB accumulation where the culture showed increased PHB synthesizing ability, which amounts to maximum PHB production. Intracellular accumulation of PHB seems to be related to the ability of the algae to grow in the presence of acetate. Normally the PHB production of the organism was induced by α-ketothiolase enzyme. The enzyme was inhibited by free coenzyme A. However in the presence of high amount of acetyl CoA the inhibitory effects of coenzyme A is overcome. The addition of sodium acetate to medium will monitor the acetyl CoA activity. Hence the medium was optimized by using sodium acetate (Massimo Vincenzini, 1990). *Pseudoneochloris marina* was not able to produce PHB under 100% nitrogen deficient conditions. Growth was observed under 50% nitrogen deficient conditions and maximum growth occurred in media with 100% nitrogen. Although PHB synthesis is enhanced under nutrient limiting conditions but certain algae may not be able to produce high amount of PHB at very low nitrogen concentrations (Neelam Naranje *et al.,* 2016). The optimum pH for the production of PHB was found to be around pH 6-10. Results of the present study on pH was matched with that of (Grothe *et al.,* 1999) where they found out that the initial pH value of 6-10 gave the best PHB yield whereas the pH values that changed even slightly from the optimum reduced the culture performance in accumulation of PHB to a extent. Rice boiled water was found to be a suitable inexpensive substrate for the production of PHB (Castilho *et al.,* 2009). On the other hand, for PHB production, use of inexpensive renewable carbon sources such as waste materials is essential to reduce the production cost further. Cheaper raw material and its efficient use have direct impact on the final price of the product in market.

CONCLUSION

At present, PHB production from photosynthetic microorganisms to produce biodegradable plastics as an alternative to conventional plastics is considered to be cost effective and eco-friendly compared to bacterial production of PHB. *Pseudoneochloris marina* do have the potential to produce biopolymers like PHB from Co_2 as the sole carbon source, and the yield of PHB could be increased by various nutrient limiting conditions etc. The technology routes for the production of algae-based bioplastics are still under the research phase and are far from commercialization. Algal based bioplastics can play a vital role as an environment friendly and biodegradable alternative compared to conventional plastics. The main drawback of PHB commercialization is its high production cost which is 10 times higher than that of synthetic plastic. So, the present research work mainly focused on the fermentative production of PHB by P*seudoneochloris marina* using low cost raw materials like rice boiled water, Jackfruit seed powder etc. as substrates.

References

1. Falcone DB. "Biodegradable Plastic in the environment." MSC Thesis, UFSCar, Sao Paulo, Brazil; 2004.
2. Greg Stevens. How to make algae bioplastic . 2010. URL: http:// green-plastic.net
3. Khanafari, A. Akharan Sepahei, M. Mogharab. Production and Recovery of Poly-ß-Hydroxybutyrate (PHB) from whey degradation by Azotobacter. Iran. J. Environ. Health. Sci. Eng. 2006; 3(3): 193-198.
4. Anthony G. Ostle and J.G. Holt. Nile Blue A as a fluorescent stain for Poly-β- Hydroxybutyrtae. Applied and Environmental Microbiolgy. 1982; 44(1):238-241.
5. Yavuz Beyath and Belma Asim. Determination of PHB production in different growth medium by cyanobacteria. Pakistan Journal of Biological Sciences. 2006; 9(1):45-47.
6. Alejandra de Almeida *et al*. Effect of Aeration on the synthesis of Poly (3Hydroxybutyrate) from glycerol and glucose in recombinant *Escherichia coli*. Appl. Environ Microbiol. 2010; 76(6):2036-2040.
7. Lee, S.Y., Lee, K.M., Chang, H.N. and Sreinbuchel, A. Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly (3- hydroxybytyric acid) and morphological changes. Biotechnol. Bioengg.1994; 44: 1337- 1347.
8. D. Byrom. Polymer synthesis by microorganisms: Technology and Economics. Trends in Biotechnology.1987; 5(9):246-250.
9. Uma Maheswari N. and K. Ahilandeswari. Production of bioplastic using *Spirulina platensis* and comparison with commercial plastic. Research in Environment and Life Sciences. 2011; 4(3):133-136
10. Kanokphorn *et al*. Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. Electronic Journal of Biotechnology.2008; 11(3):1-12.
11. Sundaramoorthy Balaji, Kadiyala Gopi Balaji Lavanya and Bhaskaran Muthuvelan . Isolation and optimization

of Poly- β- hydroxybutyrates producing Cyanobacterial strains. International Journal of Applied Biology and Pharmaceutical Technology. 2011; 3(1): 137-145.
12. Onder Uysal, Kamil Ekinci. The effects of nitrogen deficiency on the production of Polyhydroxybutyrate (PHB) using *Spirulina platensis*. Luccrari Stiintifice. 2016; 59(1)
13. G. Mahitha *et al*. Microbial Polyhydroxybutyrate production using cheap raw materials as substrates. Indian Journal of Pharmaceutical and Biological Research. 2016; 4(1):57-62.
14. Misra *et al*. Screening of poly-β-hydroxybutyrate-producing microorganisms using Fourier transform infrared spectroscopy. Biotechnology letters. 2000; 22(15):1217-1219.
15. Massimo Vincenzini, Claudio Sili, Roberto DePhillippis, Alba Ena and Richardo Materassi . Occurrence of Poly-β- hydroxybutyrate in Spirullina species. J. Bacteriol. 1990; 172: 2791-2792.
16. Neelam K. Naranje, Bharat J. Wadher , Manohar G, Muddeshwar . Utilization of low cost carbon substrates and optimization studies for maximum yield of polyhydroxybutyrate from B. megaterium. International Journal of Current Microbiology and Applied Sciences. 2016; 5(11):623-630.
17. Grothe, E.; Young, M.M. and Chisti, Y. Fermentation optimization for the production of poly (β-hydroxybutyric acid) microbial thermoplastic. Enzyme and Microbial Technology. 1999; 25(1-2): 132-141.
18. Leda R. Castilho *et al*. Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. Bioresource Technology. 2009; 100(23):5996-6009.

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