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Studies on Polyhydroxybutyrate (PHB) Produced by *Halomonas Salifodinae*

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Abstract:

Halophiles are extremophile organisms, or in other words a category of extremophiles that thrive optimally under high concentrations of salt. The term Halophile has been derived from Greek words meaning 'salt-loving'. In present study highly PHB producing ability of the halophilic *Halomonas Salifodinae* was isolated from juhu beach Mumbai, MS India. Polyhydroxybutyrate is one of the best ecofriendly biopolymer and alternative of synthetic plastic material. It is synthesized by various organisms which is a reserve food material used by microbes as an energy as well as carbon source. Polyhydroxybutyrate shows brittle, biodegradable, environment friendly properties. In present work isolates was produced Polyhydroxybutyrate by using Nitrogen deficient medium and modified medium with mannitol as carbon source. The maximum PHB production was observed in optimized different parameters in Marine Medium submerged fermentation medium. Large scale production was

further scale up in 4 L bioreactor for 5 days of incubation period. The Extraction of bacterial PHB was done by previously described sodium hypochlorite *John and ralpha* method. Selected bacteria species produced PHB was molecularly confirmed by FTIR & NMR analytical methods.

Keywords: Polyhydroxybutyrate, Halophiles, *Halomonas Salifodinae,* Marine Medium, *FTIR, NMR*.

Introduction

The use of plastics, replacing materials such as paper and glass, has become a staple of modern society. Plastics tend to offer high durability, good mechanical properties, and low production cost. However, the plastic's durability also becomes an issue once it has been discarded. Conventionally there are two main ways to treat plastic waste, recycling and incineration, both of which have their disadvantages and issues. The use of non biodegradable plastics causing the environment pollution, hence biodegradable plastics have emerged as a useful alternative to overcome the environmental pollution. During the last decade, environmental pollution and exhaustion of non renewable resources have created much interest in natural materials like Poly - hydroxybutyrate as a biodegradable. The physical properties of the PHB are similar to those of some conventional plastic [Howells, E.R 1982]. Because of their good biodegradability and biocompatibility PHB have attracted interest in their use as an alternative to petroleum based plastic including fine chemicals, plastics, printing materials bio fuel, agriculture, marine, medical and other fields [Doi, Y 1990].

Poly (_-hydroxybutyrate) (PHB) is an intracellular storage compound, which provides a reserve of carbon and energy in microorganisms [Anderson, A. J. and Dawes E. A 1990]. It accumulates as distinct inclusions in the cell and comprises up to 60% of cell dry weight for strains of *Halomonas Salifodinae*, under conditions of nitrogen and phosphate limitation and

excess of carbon source. PHB, which is a biodegradable, biocompatible thermoplastic, has broadly similar physical properties to polypropylene. It has many applications in medicine, veterinary practice, and agriculture due to its biodegradability [Howells E 1982]. Currently the main problem, which limits the widespread use of PHB and its copolymers, is its relatively high cost compared to polypropylene. The fermentation process, substrates and product recovery are the major costs [Byrom 1987]. Research has focused on reducing these costs by optimizing fermentation.

In our previous study isolation of PHB from different microorganisms with different optimizing media in these research studies PHB was produced by halophilic *Halomonas Salifodinae* species. Microorganisms that love salt are known as halophiles. Halophiles are extremophile organisms that thrive in environments with very high concentrations of salt (Mohsin Azhar et al 2014). In recent years, uses of halophilic microorganisms have significantly increased. Many enzymes, stabilizers and valuable compounds from halophiles may present advantages for the development of biotechnological production processes. The present research work was carried out to optimize physical process variables viz; temperature, pH, salt concentration, carbon source, nitrogen source and agitation speed for enhanced PHB production under submerged fermentation by *Halomonas Salifodinae* using mannitol and yeast extract as potent carbon and nitrogen source. Shake flask cultivation was further scale up in 4 L bioreactor. The microbial PHB was extracted by previously described *john and ralpha* used method. The extracted PHB was analytically characterized by FTIR and NMR technique it was confirmed with reference PHB purchased from sigma Aldrich Bangalore, India.

Methodology

1.1 Bacterial Strains

Halophilic bacterial strains were isolated from sea water Juhu beach Mumbai in Maharashtra India. The water sample was serially diluted and the individual colonies were further sub-cultured to obtain pure bacterial isolates which were then screened for the production of PHB by primary and secondary screening methods. Four bacterial strains were found to produce PHB which were biochemically characterized and molecularly identified by 16s rRNA sequencing as *Halomonas Salifodinae, Bacillus licheniformis, Halomonas shengliensis and Alishewnella jeotgali* certified by NCL CSIR Pune. The PHB produced by each of the strains was evaluated by previously described John and ralpha 1961 applied method and the most efficient strain with maximum polymer production was found to be *Halomonas Salifodinae* which was later labeled as MA1.The strain was maintained on agar slants and stored at 4ºC until use.

1.2 **Composition of the culture medium**

For PHB scale-up, Marine medium (MM1) containing the following composition (g/L): NaCl: 150gm, KCl : 0.7 gm, MgSO4 : 13.4gm, Mannitol : 30gm/l, yeast extract : 2gm/l all elements were sterilized separately at 121°C for 20 min. The pH of the medium was maintained at 8.0.

1.3 **Lab level study of PHB production under Submerged Fermentation (SmF)**

Prior to scale-up, a shake flask study was carried out using the efficient bacterial isolate by growing it in 100ml of Marine medium to evaluate its growth pattern and PHB production for up to 5 days. The optimization for maximum PHB production by the selected isolate was carried using MM1 as the culture media to evaluate several cultural parameters and to determine their

effect on growth and PHB production. The optimized value for each parameter was selected and kept constant for further experiments. There are several cultural parameters such as; temperature, pH, carbon, nitrogen source and their ratio, salt concentration, agitation speed and inoculums size etc.

1.4 **Scale-up production of PHB**

For large scale production, a loopful pure culture of *Halomonas salifodinae* was taken from a freshly streaked plate and inoculated into a test tube containing 3 ml of nutrient broth. The inoculated tube was incubated at 40ºC in an orbital shaker at 120 rpm until an optical density of 2.0 OD 600nm was attained. The pre-inoculum culture was transferred into 250 ml of Marine broth taken in a 500 ml conical flask. It was incubated at 40ºC in an orbital shaker at 120 rpm for about 48 hours until a growth of 6.0 – 8.0 OD 600nm was attained. The fermentation process was studied in a 5 L fermenter (D.S.M. college parbhani) with a working volume of 3.5 L using the optimized parameters mentioned above and production medium. Initially, 3500 ml of Marine broth was prepared was and added into the fermenter and the medium was sterilized in situ. Manitol was added as a carbon source at 3% (w/v) concentration to maintain the overall ratio of carbon and nitrogen sources at 3:0.3%. The aeration and agitation were maintained at 1vvm and 500-700 rm respectively. The Dissolved oxygen level was not controlled. The impeller speed was maintained at 120 rpm and aeration was set at 1vvm. The pH was maintained at 8.0 using 1 N NaOH and throughout the process temperature was kept at 40°C. 0.5 mL of the antifoaming agent, soybean oil was initially added to the fermenter. The culture broth was taken 5 days throughout the whole fermentation run and about 20 ml culture broth was taken every 6 hours throughout the whole fermentation run and analyzed for cell dry weight (CDW) and PHB yield.

1.5. **Extraction of PHB:**

After 5 days of incubation, fermented broth was taken and centrifuged at 6000 rpm at 4°C for 15min.The supernatant was discarded and take pellet. Extracted pellet was added 10% of sodium hypochlorite in it and incubated it for 1 hr. at RT. After the incubation period the tube with mixture was again centrifuged at 8000 rpm at 4°C for 15 min. Discard the supernatant and take pellet. Wash the pellet by acetone and ethanol in (1:1 ration). Take pellet & dissolved the pellet in 10% of boiling chloroform. After the pellet dissolved in chloroform, Whatman filter paper was used to filter out the cell residues so that only PHB is present in the chloroform solution. The chloroform was evaporated and PHB powder was collected and stored at RT for further analysis. Calculate the amount of extracted PHB using below formula. (Haliru musa et.al 2016).

Percent production of PHB was calculated by using the formula:

% of PHB = Total weight of PHB / Total weight of pellet x 100

1.7 Characterization of PHB:

1.7 1.0 FTIR

Approx. 1mg of sample mixed with approx 100 mg of KBr & mixed homogenously with the help of mortar & pestle. Make the thin pellet using a hydraulic pellet press. Analyze the sample using the SAIF IIT Bombay applied FTIR technique & collect the spectra. It analyzed through Burkey Germeny Model 3000 Hyperion Microscope with Vertex 80 FTIR System

1.7 1.2 NMR

^{1}H NMR 600 proton spectra was carried out by dissolving the pinch of purified PHB and extracted crude PHB in deuterochloroform (CDCl3). Homogenize the mixture to make the uniform solution. Analyze the sample using the SAIF IIT Bombay applied NMR analytical instrument & collect the spectra.

Result and Discussion:

Isolation and screening of the PHB producing Halophilic bacteria:

After several dilutions and sub culturing in the liquid as well as solid medium, colonies were isolated in the enrichment medium containing Maximum 15% NaCl. Halophilic PHB producers was primarily screened by sudan black B staining method and secondarily confirmed by TEM. In previous study the spcies *Halomonas salifodinae* was isolated from Egyptian Mediterranean salt lakes in these study it was isolated from Mumbai juhu beach Maharashtra India. In this research study it was screened by Sudan Black B staining method. In some study it was screened out using sudan Black B staining and some other method like nile blue staining method. In TEM irregular bodies of inclusion were observed in previous study same result was observed. In previous study the obtained image were using different image detector software's. In this study TEM results were examined using Bact_nande image detector software.

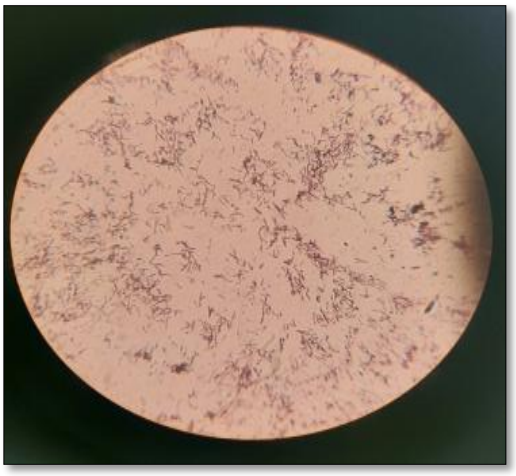


Fig: Sudan Black Staining of PHB

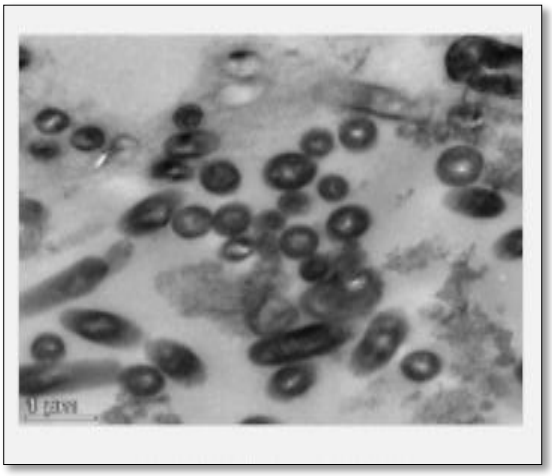


Fig: TEM analysis of PHB

Shake flask study and optimization of growth parameters

The bacterial isolate was grown in Marine Medium to study the growth pattern of each strain and to determine the yield of PHB. The maximum PHB production was observed in rich amount of carbon and decreased amount of nitrogen source ratio contained MM1medium. Halomonas *salifodinae* was shown that the maximum growth was attained on the fifth day at 40°C temperature. In previous study selected organism was optimally growing on 37ºC temperature and pH 7.0 for 7 days of incubation period.

Fig: Shake Flask Study of Fermentation.

In these studies it was optimally growing on 40°C temperatures and 8.0pH for 5 days of incubation period. In previous study the optimized media with using different carbon and nitrogen source in this study optimize the production media with mannitol as carbon source and yeast extract as nitrogen source.

Reactor studies for large scale production of PHB

In the fermentation study of PHB production using mannitol as a carbon substrate, it was observed that the maximum PHB production was attained at 96 hours of growth after which a decline in yield was observed. The temperature and pH was maintained throughout the fermentation process. The D.O. level was not controlled but was allowed to fall freely with 20 % saturation until equilibrium was established. It was seen that there was an increase in the Cell Dry Weight (CDW) as the specific growth rate increased at every 6 hours interval. At the end of 96 hours, the CDW was found to have increased with increase in the PHB production. The CDW decreases and PHB production also declined after 120 hours. In previous study fermentation was also carried in glass bioreactor with different volume of media, composition of media with different microorganisms (Swetha Narayankumar et.al 2019)

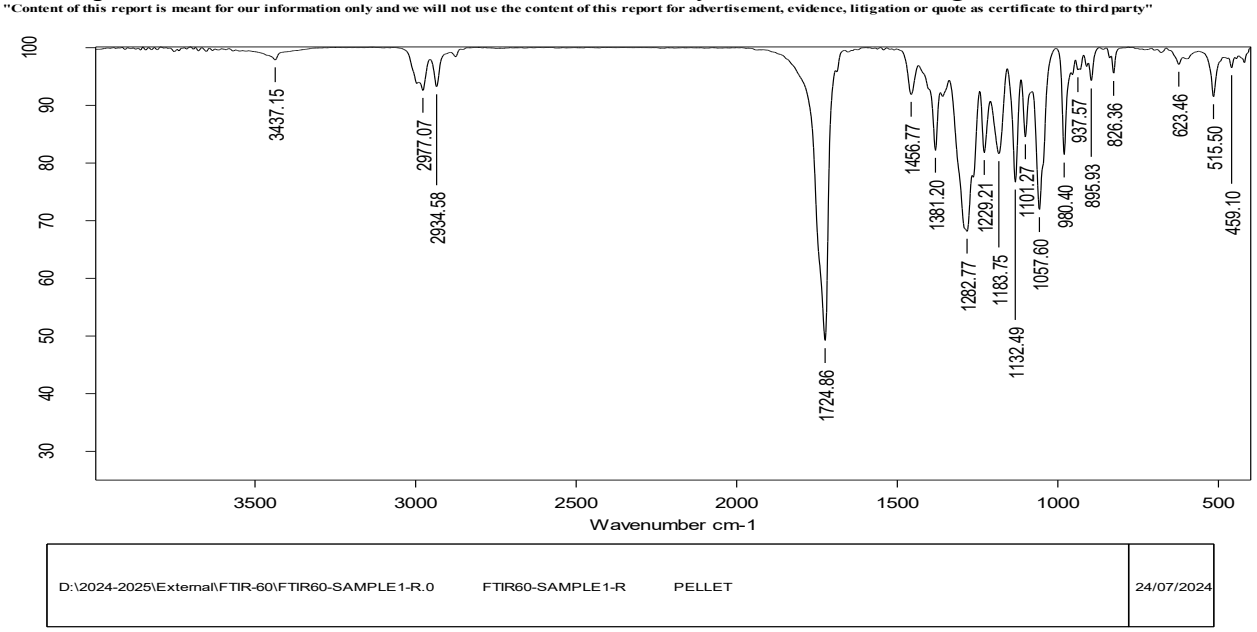
Fig: Large Scale study in Glass Bioreactor.

Production and extraction of PHB

PHB was synthesized by Halomonas *salifodinae* as described above using accessing of carbon source (3% of mannitol) and minimal concentration of nitrogen source (0.2 g/1yeast extract). After incubation for 5 days, the cells were harvested and the cell dry weight was measured. The PHB that accumulated in the cells was extracted using chloroform. The increased ratio of carbon and decreased ration of N2 source was increased the amount of PHB the same results was fined in previous study. In previous study it required different time of incubation period of PHB production in this study it required 5 days for optimum PHB production.

FT-IR of PHB from Halomonas salifodinae

Figure A. Demonstrates FT-IR spectrum of the purified PHB and B. for extracted sample. The extracted sample peak at 1724.94 cm−1 indicates to ester carbonyl group of PHB. Moreover, the peak at 1283.14 cm−1 is due to the -CH group in the biopolymer. The same band was obtained in previous study (Bayari and Severcan, 2005). The peak at 2934.76 cm−1 corresponds to the stretching and deformation vibrations of the O-H groups. The presence of the peak at 2977.04 cm−1 may be due to the C-H...O hydrogen bond. The specific peak at wave numbers 3437.42 cm−1 attributes to the terminal O-H bonding or water adsorption on the PHB (López *et al*., 2012). The result was resembled to previous study. The FT-IR spectrum is very closer to the FT-IR spectra of PHB extracted from *Halomonas salifodinae,* K5, *B. megaterium* and commercial



PHB (Liu *et al*., 2014; Dhangdhariya *et al*., 2015). The same result was obtained in previous study and slightly changed the band absorption rate.

Fig A : FTIR Analysis of Standard Reference PHB

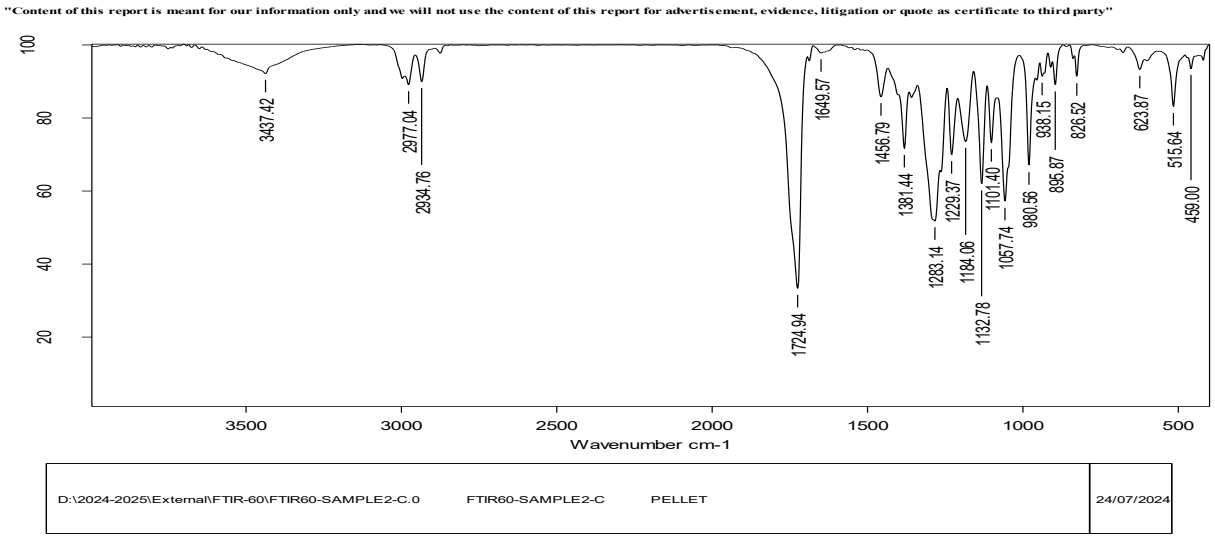


Fig B: FTIR Analysis of Extracted Crude PHB

NMR of PHB from Halomonas salifodinae

Figure A. Demonstrates FT-IR spectrum of the purified PHB and B. for extracted sample A selected ^{1}H NMR spectrum of crude extract showed the presence of three main groups of signals, characteristics for PHB: a doublet at 1.29 ppm which is assigned to the methyl group, two doublet of doublets, at 2.45 and 2.60 ppm, for the methylene protons and a doublet of quadruplets at 5.25 ppm, characteristics of the methane group. The structure of the extracted PHB was confirmed by comparison with a ^{1}H NMR spectrum of a commercial PHB sample and previous study (María Eugenia Patiño Iglesias et. al 2014).

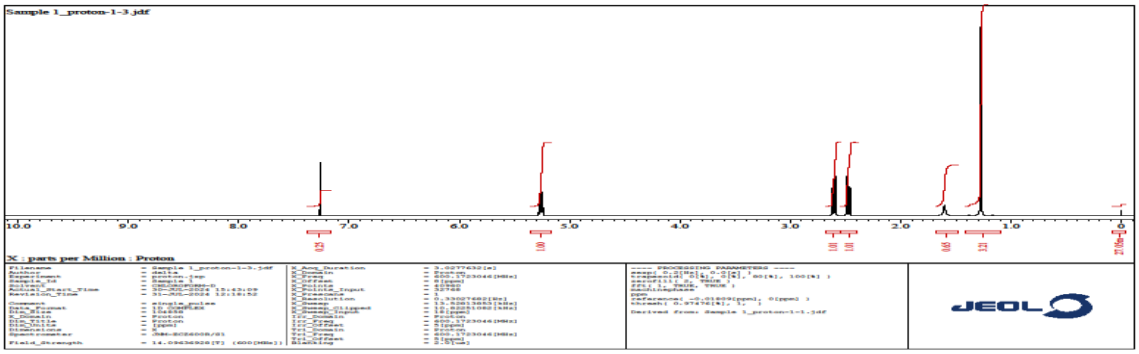


Fig: Fig A: NMR Analysis of Standard Reference PHB

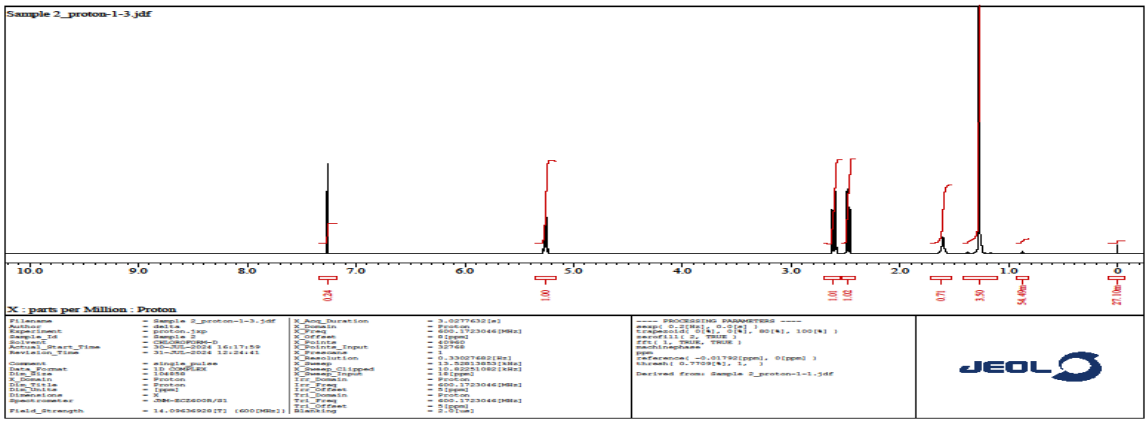


Fig B: NMR Analysis of Extracted Crude PHB

Conclusion:

Polyhydroxybutarate (PHB) was derived from the novel halophilic isolates of *Halomonas salifodiane* has been isolated from the Juhu Beach Mumbai Maharashtra India. *Halomonas salifodiane* can produce higher yielding of PHB, supplemented with 3% (w/v) Mannitol and 0.3% (w/v) yeast extract, respectively. It further scales up of production using 4 L of glass bioreactor. The extracted polymer was conformed to FTIR and NMR analytical techniques

through SAIF IIT Bombay Maharashtra, India. It resemble to commercial PHB purchased from sigma Aldrich Banglore India.

Acknowledgment

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