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Screening and optimization of Halophilic strains for Polyhydroxybutyrate production isolated from Sambhar Lake, Rajasthan

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Abstract

Poly-β-hydroxybutyrate (PHB) is gaining importance in advanced material research through its application in the field of bioplastic material and medical field applications. Production of PHB has been optimized in bacterial species. The current work focuses on isolation characterization of halophilic bacterial strain from Saltern Sambhar lake, Rajasthan and optimization for high production of PHB. The optimized growth was studied in different media Nutrient broth, M9, HSM and GM 2. Modified DSC-97. Further optimization studies were carried out in different pH (7-10), sodium chloride concentrations (1-5 M) incubation time (24, 48, 72, 96 and 120 hrs) and variable carbon sources (glucose 1-4%). PHB was extracted by chloroform extraction protocol and was detected and characterized by TLC and FTIR. Halophilic bacterial strain was identified through 16s RNA sequencing method.

Keyword: Poly-β-hydroxybutyrate, Modified DSC-97, Optimization, Bioplastic etc.

INTRODUCTION

Bioplastics are major substitutes of synthetic plastics which are easily degradable by environmental factors and through various types of micro-organisms such as *Alcaligenes sp. and Pseudomonas sp.* [1]. Bioplastic material are majorly categorized with PHA and PHB classes which are basically polyesters of hydroxyl acids synthesized by various microorganisms as energy and food reserve material under environmental stress conditions such as limitation of nutrients (N, P, K, O) and excess of carbon source [2, 3]. It was accounted that production of PHAs and its cell dry weight (cdw) was upto 90% [4]. Although the optimized conditions and the production of PHB is not yet commercialized due to high production cost [5, 6]. The member of PHA class i.e. Polyhydroxybutyrate has high crystalline nature and low strength, which exploits its applications at industrial level. Due to which preparation of nanocomposites increased the interest of scientists in the field of nanotechnology.

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Polyhydroxybutyrate (PHB) is the most widely studied type of PHAs reported for the first time by Lemoign 1923 in *Bacillus megaterium*. Among all of member of PHA family, poly-β-hydroxybutyrate is the most common biodegradable polymer and a promising alternative to synthetic non-degradable or petroleum- based plastics.

The strategic optimization parameters should be considered for increasing the production levels of PHB and thus efforts are required for improving the quality of product [7]. Much efforts are being required for reducing the production cost of polymeric materials [8,9] or develop (genetically engineered) strains [10] capable of high level PHB production. The present work mainly focuses on the identification of halophilic strain capable of producing PHB and optimization of culture conditions for halophilic bacteria by using various synthetics lab medium (M9 medium, DSC-97 medium, Nutrient Broth, HSM and GM2) as substrate and further the large scale production and characterization of PHB samples.

MATERIAL AND METHODOLOGY

Collection of Samples & Isolation of halophiles

Satellite view of Sambhar Lake

Sambhar Lake is located in Nagaur and Jaipur districts of Rajasthan. Lake covers an area around 190 to 230 square kilometer. It was analysed and reported that Rajasthan is the third largest salt producing state. A total 38 villages are located near to the Sambhar Lake.

Lake water sample were collected in autoclaved flasks and transferred to laboratory at 4^{o}C. For isolation of microbes from water sample, one milliliter of water sample was spread on nutrient agar plates with defined salt concentration (2-3 M). Plates were incubated at 37^{o} C for 48 to 72 hrs. Morphologically dissimilar colonies were purified and preserved on nutrient agar plates at 4^{o} C.

Staining of Intracellular Lipids

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Intracellular lipids were analysed by using Sudan Black B stain [10]. The previous studies showed that bacterial cytoplasm was stained as pink in color whereas the inclusions are black bluish in color [11, 12]. The bacterial strains were streaked on specific medium utilizing the appropriate salt concentration and supplemented with solution of Nile red dye to observed the fluorescent colonies under UV light [11].

Optimization of Bacterial growth for PHB production

Optimization on Modified DSC-97 medium

The selective media consisted of: modified DSC-97 salt with a concentration as per the paper [13]. The DSC-97 consisting of different molar concentration of salts and further growth curve analysis were done in order to optimize the salt concentration and incubation time required for the maximum growth of bacterial strains.

Optimization of bacterial isolates on other parameters (pH, NaCl conc., Incubation time and glucose conc.): Bacterial isolates were evaluated at different pH (7-10) (Singh et al., 2011), sodium chloride concentrations (1-5 M), incubation time (24, 48, 72, 96 and 120 hrs) [11] and varying carbon sources (glucose 1-4%) to analyze the growth conditions for maximum growth. Further, bacterial growth was analyzed at an absorbance at 600nm.

Extraction of PHB

The extraction of PHB was done by centrifugation and further harvest the cells (7000rpm/15min), and the pellet obtained was digested with sodium hypochlorite at 37°C for 1-2 hrs, followed by extraction with chloroform and precipitation with ethanol and acetone (1:1). Further, chloroform was evaporated at 29°C to get the crystal of PHB [15, 11].

Detection of PHB production by bacterial isolates using thin layer chromatography

40 µl of PHB sample (dissolved in chloroform) was loaded on TLC plate and plate was kept in benzene and ethyl acetate chamber (1:1) for 30 minutes. Staining was performed, with the vapor of iodine solution (20ml) at 80-90°C in water bath for 5-10 min. Staining causes formation of green-black colored spots after a time lag of around 15 minutes on TLC plate. The spot indicates the presence of PHB [16, 17].

PHB production on bench scale Fermenter by using optimized conditions done on small scale

Bioreactor Experiment

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The fermentation was carried out aerobically on a bench scale fermenter manufactured by BioGen India Ltd. The fermenter vessel containing 2.5 liter of different medium solution was sterilized in the autoclave at 121° C for 15 minutes. The solution (NB or DSC-97) in the fermenter vessel was allowed to cool and was inoculated with 250 ml of actively growing inoculum. Aerobic condition was provided with 150 rpm agitation speed at pH 9.

Fermentation conditions for PHB production

Fermentation Condition for Nutrient Broth

The solution of Nutrient Broth with 3M salt concentration is used for the production of PHB at different incubation time (24, 48 and 72 hrs). The fermenter was agitated at 150 rpm and fermentation was carried out at 37°C and pH 9. Samples collected were centrifuged and the pellet was analyzed for biomass and PHB concentration.

PHB production

Fermentation process was used for PHB production at large scale. Fermenter vessel (Bioage, Lab No. 29-403, LPU) was autoclaved with 2.5 liter of NB and modified DSC-97 media with different time intervals. Isolated strain was inoculated (250 ml) in fermenter vessel. Aerobic condition was provided with 150 rpm agitation speed at pH 9.

Characterization of PHB

FTIR technique was used for rapid identification of PHB obtained from isolates. 1 gram of PHB powdered sample and a pellet of potassium bromide was prepared for analysis. The SHIMADZU FT-IR equipment facility in Chemistry department LPU, was used for the characterization of pure PHB by analyzing their functional groups [18, 1].

Result and Discussion

Isolation of halophiles from the production of PHB

1 ml of water sample was spread on NB plate containing 1M, 2M, 3M, 4M and 5M salt at pH 7, pH 8, pH 9 and pH 10. Petriplate was incubated at 37°C temperature and 150 rpm shaking speed.

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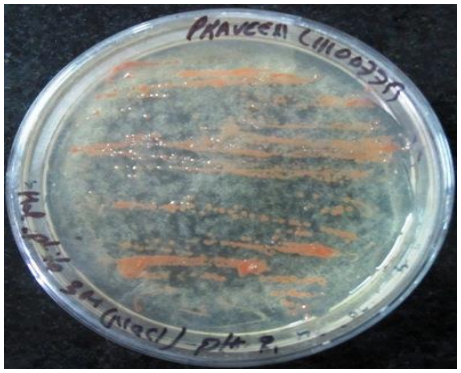

Fig 1: Streaking of bacterial strain by streak plate method

Screening of halophiles for PHB granules

- **Screening by Sudan Black B stain**

Isolated strain was stained with Sudan Black B dye on glass slide with microbial smear and result was observed under the microscopic view at 100X (Magnus Microscope LPU).

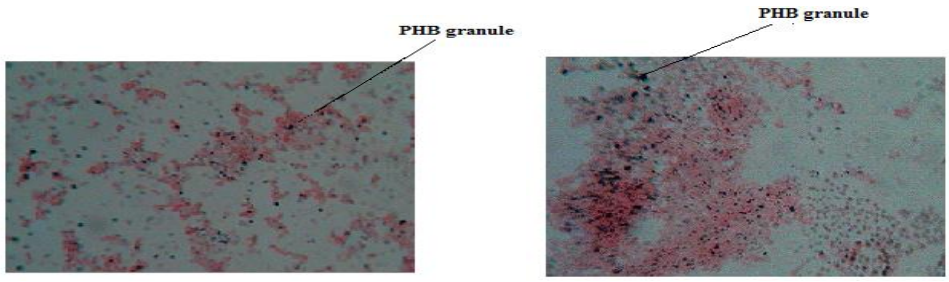

Fig 2: PHB granules are stained by Sudan Black B

- **Screening by Nile Red A stain**

Isolate were streaked on petriplates containing DSC-97 media with 3M salt and Nile Red A dye at 150 rpm and incubated at 37˚C temperature. The result was observed under UV light on UV illuminator.

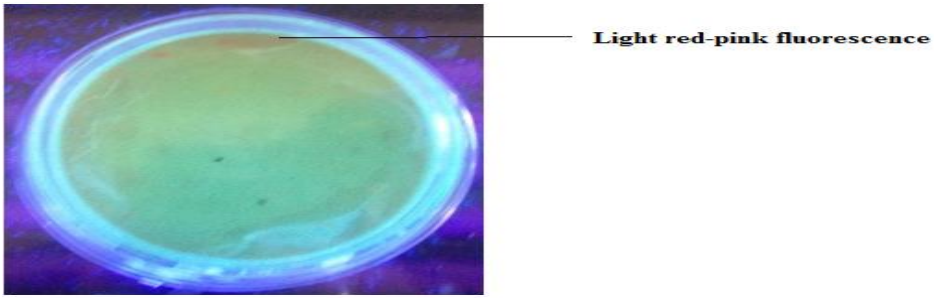

Fig 3: Nile Red A staining of isolate under UV

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Optimization for PHB production

Isolated strain was optimized on four reported medium with different salt concentration and at variable pH. Suitable medium for isolated strain were selected and optimized at different time intervals with shaking speed at 37° C for PHB production. The growth of isolated strain was measured by spectrophotometer at 600 nm wavelength.

Media Optimization for PHB production

pH optimization for PHB production for DSC-97

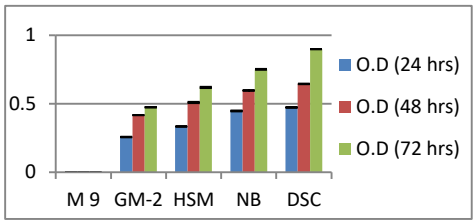

Fig 4: Spectrophotometer analysis (OD) values after 24, 48 and 72 hrs in different laboratory medium

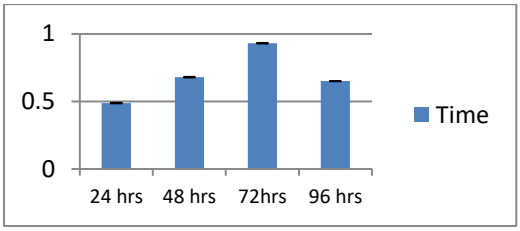

Fig 5: Spectrophotometer analysis (OD) value in different time intervals at 600 nm (OD)

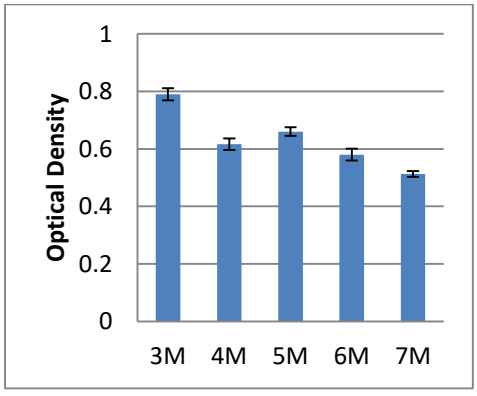

Fig 6: Growth of isolates at different salt concentrations in modified DSC-97 media

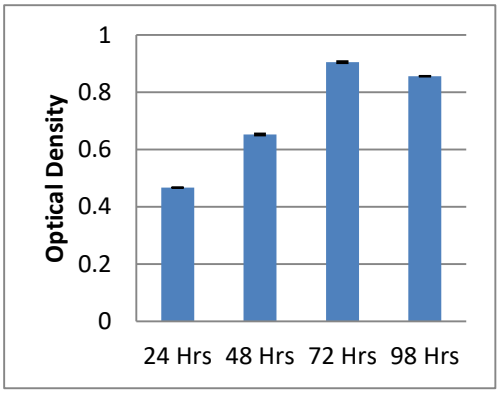

Fig 7: Spectrophotometer analysis in at different incubation time in DSC-97 media

- **Carbon Source Optimization for PHB production**

(i). Optimization at different glucose concentration

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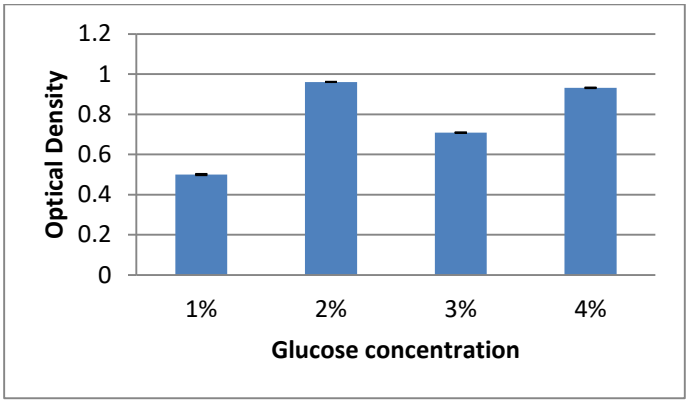

Fig 8: Growth of isolates at different Glucose concentration as carbon source in DSC-97 (72hrs)

- **PHB Sample (Extracted PHB) by Sodium Hypochlorite Method**

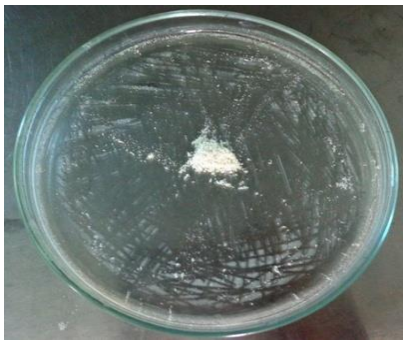

Fig 9: Extracted PHB in powder form

- **Determination of PHB**

Thin layer chromatography was performed for the determination of PHB. The mixture of silica gel and calcium sulphate was poured on the glass plate. TLC plate with PHB sample was kept in benzene and ethyl acetate (1:1) solvent chamber for 40 min. TLC plate was kept over the beaker containing iodine solution (50 ml) for 5-10 min in order for it to get saturated with iodine vapour. After 10 min yellow- green color spots indicated the presence of PHB. The Rf value was measured [19] (Sharmila et al., 2011).

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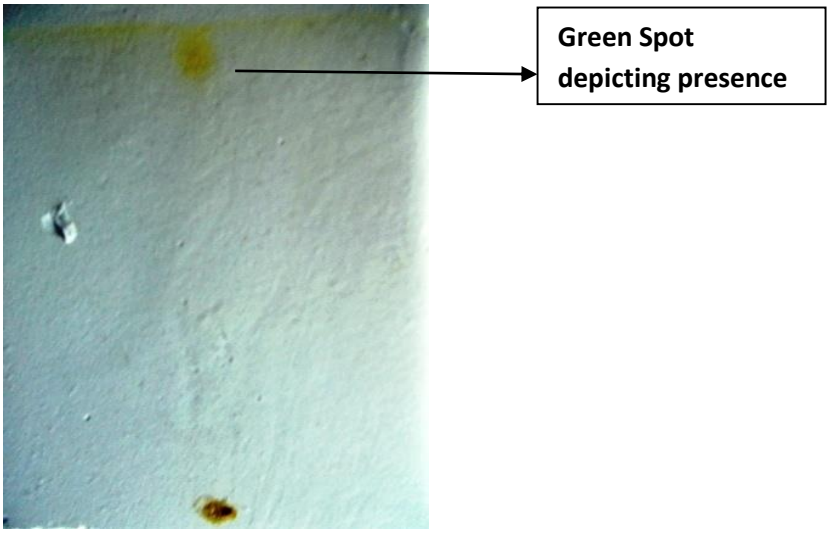

Fig 10: Thin layer chromatography of extracted PHB

The green black spots indicate the confirmation of presence of PHB. The Rf value obtained was 0.76 which indicates that extracted compound is PHB.

Characterization of PHB

FTIR technique was used for the characterization of PHB.

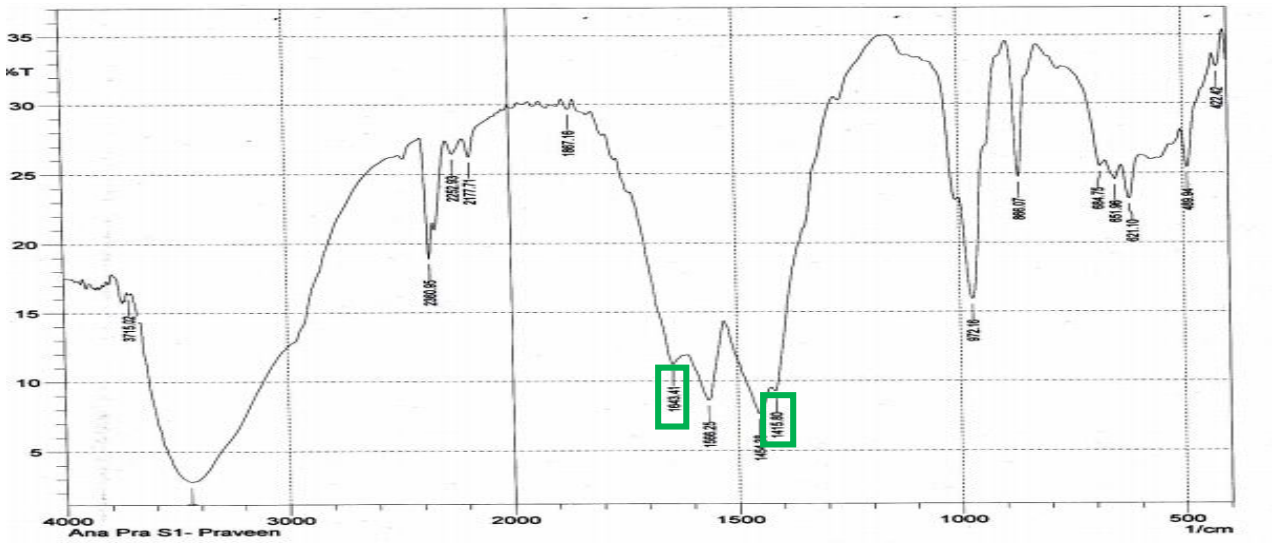

Fig 11: Characterization of PHB in DSC-97 after 24 hrs incubation in fermenter

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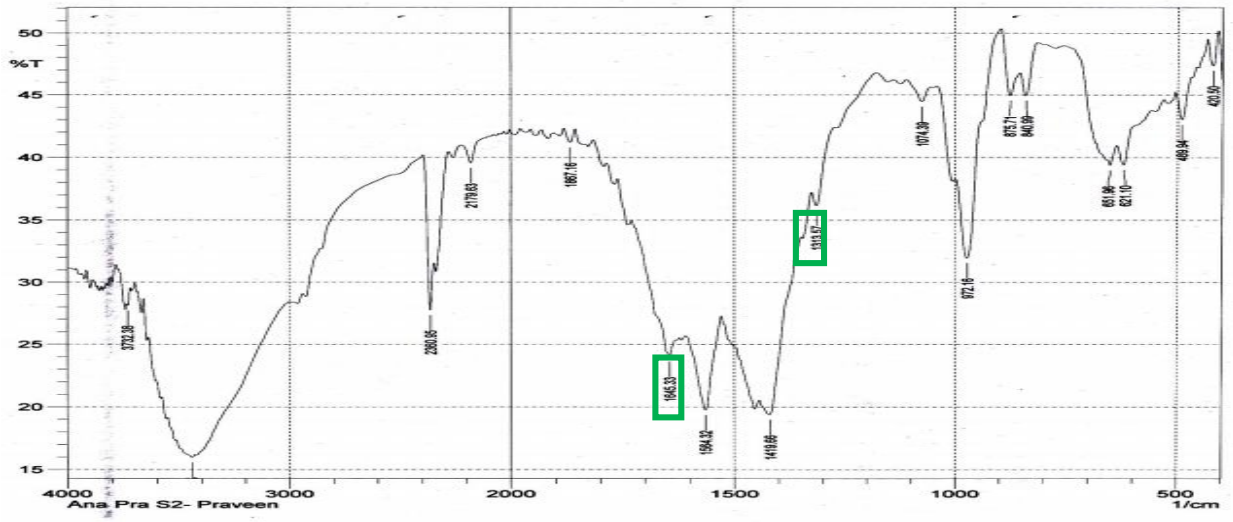

Fig 12: Characterization of PHB in DSC-97 after 48 hrs incubation in fermenter

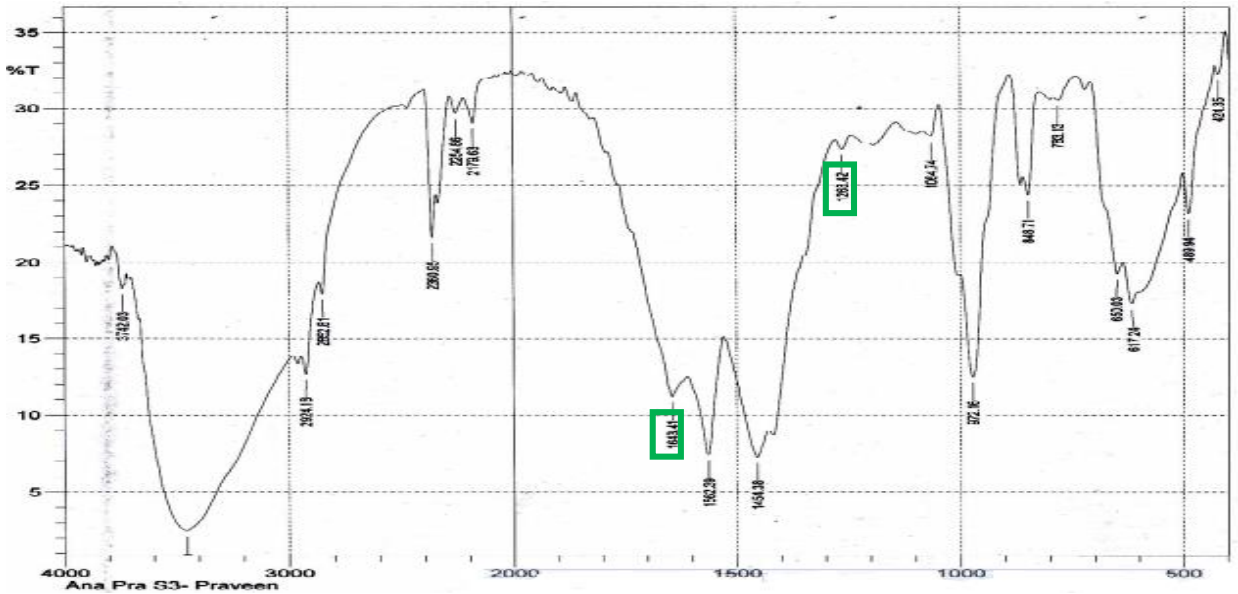

Fig 13: Characterization of PHB in DSC-97 after 72 hrs incubation in fermenter

IR study was carried out for the PHB produced from DSC-97 medium, the peak value observed for the PHB of isolate sp. were 1643.41 cm^{-1} and 1274.99 cm^{-1} (in DSC-97 after 24 hrs), 1645.33 cm^{-1} and 1313.57 cm^{-1} (in DSC-97 after 48 hrs.), 1643.41$cm^{-1}$and 1263.42 cm^{-1} (in DSC-97 after

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72 hrs) which was near to the peak value of pure PHB (1724.03 cm^{-1} for C=O and 1280.73 for C-O). So this indicates that the isolated compound may be PHB.

- **16s RNA for identification of isolate**

The microbial strain was identified by 16s RNA technique for molecular characterization. The strain was identified as *Halobacterium* sp. RD 1 All the amplified sequences were then compared with nucleotide sequences already available in NCBI-BLAST.
The sequence was compared eith GenBank database and showed 100% similarity with the sequence of *Halobacterium sp. BRTH1*

Phylogenetic dendrogram

The phylogenetic dendrogram showed that the isolate lies within the cluster and the phylogenetic position is to be most similar (100%) to the sequence from *Halobacterium sp. BRTH1*

Discussion and Conclusion

Isolation and characterization of halophiles from saltern lakes and water bodies is of considerable importance. Production of PHB from halophiles has been tried by many workers earlier as well. Halophiles are subjected to extreme salt stress and desiccating conditions which induce them for the production of intracellular lipid and PHB material. Conditions at Sambhar lake are not only highly saltern but also highly alkaline, so halophilic bacteria isolation from Sambhar lake involved maintenance of high salt as well as high pH in the nutrient medium. Various specialized media for halophiles established earlier by different researchers were utilized for the isolation procedure. DSC 97 media established earlier was modified with peptone in place of CAS amino acids and utilized for the growth and production of PHB. Nile Red A and Sudan Black B staining was performed for screening of PHB production. The PHB granules inside the bacterial cytoplasm were stained in blackish color by Sudan Black B dye. The isolated colony was stained with Nile Red A and showed light pink color in UV light.

Further, glucose modification and carbon source optimization was also performed. PHB content produced was extracted with Chloroform/Sodium Hypochlorite method.

The best growth of isolate was observed on DSC-97 (at 7g/l peptone, 2% glucose, 3 M NaCl concentration, temperature 37^0C, shaking speed 150 rpm and pH 9 after the optimization on different mediums, pH optimization, optimization of salt concentration, temperature

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optimization, shaking speed optimization, optimization of incubation time and carbon source optimization.

The isolated strain was identified as *Halobacterium sp. BRTH1*. The current work defines the production of PHB from microbial source, which provides an important aspect of halophilic bacteria that they can be optimized and modified to produce industrially important polymeric material such as PHB. The market cost of PHB material is still very high due to costly production processes. Halophilic bacterial isolates could be targeted for economic production opportunities for industrially important polymeric material such as PHB along with media optimization processes.

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Conflict of Interest

There is no conflict of interest in the research paper.

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