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Synthesis and Characterization of PHB by *Haloarcula* sp. AB19 Isolated from Salt Pans around Bhavnagar Coast

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A potential PHB producing *Haloarchaeal* sp. was isolated from salt pans around Bhavnagar coast. The isolate was identified as *Haloarcula* sp. AB19 by biochemical characterization and molecular phylogeny. PHB produced by the isolate was subjected to chemical characterization using various analytical techniques i.e., Nuclear Magnetic Resonance (^{1}H, ^{13}C- NMR) spectroscopy, Fourier Transform Infrared Spectroscopy (FT-IR), Gas Chromatography-Mass Spectroscopy (GC-MS), Thermal Gravimetric analysis (TGA) and Differential Scanning Calorimeter (DSC). The FTIR spectrum of PHB indicated presence of intense sharp blend at 1723.57 cm^{-1}, 1281.20 cm^{-1}, 2976.47 cm^{-1}, 2933.30cm^{-1}, 3448.44cm^{-1} and 1461 cm^{-1}. The ^{1}H NMR scans of the polymer revealed the presence of methyl, methylene, and methane protons. The ^{13}C-NMR spectrum showed four main peaks at 169.134 ppm, 67.608 ppm, 40.789 ppm and 19.761. The DSC results showed that melting temperature (Tm) ranged between 170 and 174 °C and thermal degradation ranged from 260 to 340 °C as revealed by TGA. Thus, the polymer identified was a monomer of hydroxybutyrate i.e., poly â-hydroxybutyric acid (PHB). Overall results suggested that NMR, FTIR, GC-MS can be adopted as a non – destructive method for its chemical characterization. Haloarchaea thus, can be exploited for the production of PHB due to its ease of recovery, reducing the production costs and providing an attractive alternative to petroleum derived plastics.

Key words: Polyhydroxybutyrate, FTIR, Magnetic Resonance, GC-MS, DSC,· TGA, Halophilic Archaea.

Halophiles constitute a very heterogeneous group of extremophiles. Success and potential of biotechnology relies on biodiversity of the molecules they produce in form of primary and secondary metabolism as a result of adaptation to their environment[1, 2]. Among the interesting products produced by halophilic Archaea is the polymer Poly β-hydroxybutyrate (PHB) which is a biodegradable thermoplastic polyester used in various ways similar to many conventional petrochemical – derived plastics currently in use and hence have gained importance[3]. It is synthesized and stored intracellularly by microorganisms as carbon and energy reservoir when grown under limitation of nutrients and excess of carbon sources[4]. The polymer has potential applications as disposable bulk material in packing films, containers or paper coatings amongst other, in medicine and agricultural forms[5]. Much effort has been devoted to reduce the production cost of PHB by improving strains and efficient fermentative and recovery processes[6]. The extreme conditions of salinity in which these organisms grow almost overrides the contamination problems. This greatly reduces the

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sterility requirements of a production facility, decreasing the investment cost[7].

NMR spectroscopy has been successfully used to study the physical properties and biochemistry of PHB. Several other methods have been developed for qualitative analysis of PHB which often require extensive and complicated sample preparation like hydrolysis, extraction, purification or methylation etc. The Fourier Transform Infrared Spectroscopy (FT-IR) method does not require extensive sample preparation and it is thus very useful for broad screening of PHB producing microorganisms[8, 9] and their cell components in intact form. It is a valuable non – destructive method for monitoring polymer formation and has the advantages of accuracy, speed and sensitivity [10, 11, 12, 13].

The present study reports the production of polymer PHB by *Haloarcula* sp. AB19, an extremely halophilic Archaeon isolated from salt pans around Bhavnagar (Gujarat) coast. The study also relates to chemical characterization of PHB using NMR, FTIR, GC-MS, DSC and TGA.

MATERIALS AND METHODS

Microorganism and growth conditions

Haloarcula sp. 1 AB19 was isolated from saltpans around Bhavnagar coast. The strain was grown and maintained on Tryptone yeast extract salt (TYES) medium with 50% glycerol [14]. The slants were stored at 4°C until use.

Strain Identification

The isolate was identified by 16S rRNA – dependent molecular phylogeny. Along with physiological characteristics like various NaCl and Mg^{2+} concentrations tolerated by the isolate, the biochemical tests performed were catalase and oxidase, hydrolysis of starch, casein and gelatin, nitrate reduction, production of indole, H_2S, ammonia and resistance against various antibiotics[15]. The 16S rRNA PCR amplification was performed using primers 8F and 1492R using BDT v3.1 and cycle sequencing kit on ABI 3730x1 genetic analyzer. Consensus sequence of 642bp 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. The sequences were aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

PHB production medium and growth conditions

To prepare seeder culture, *Haloarcula* sp. AB19 was inoculated into 250 ml capacity Erlenmeyer flasks containing 50 ml of TYES medium and incubated at 37 °C on a rotary shaker (New Brunswick Scientific Excella E24) at 180 rpm for 8 - 10 days.

10^7 cells/ml from a seeder culture were inoculated into TYES medium (500ml dispensed in 2 Erlenmeyer flasks of 1 L capacity), supplemented with higher concentration of glucose and ammonium sulphate in C: N ratio of 0.3: 1 to enhance PHB production. The flasks were incubated at 37 °C on a shaker at 180 rpm. After 12 days of growth, PHB was extracted from the pellets for further chemical analyses.

Extraction and Purification of PHB

The purification procedure for PHB produced by the isolate was performed according to the reported method[16, 17]. Cells of *Haloarcula* sp. AB19 containing intracellular polymer, were harvested from 1L of culture broth by centrifugation (Eppendorf – 5804R) at 10,000 rpm for 10min, washed once with sterile water to lyse the cells, and finally resuspended in water and lyophilized (OPERON – FDU - 7003) at -80 °C. PHB was recovered from lyophilized cells by extraction for 30h with hot chloroform in a Pyrex Soxhlet apparatus and concentrated by evaporating the solvent under vacuum. The PHB was precipitated from the concentrated solution with 10 volumes of ethanol[18] and the resulting PHB granulates were filtered twice for its chemical characterization.

Chemical Characterization of PHB

NMR-spectroscopic analysis

The ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectrum was recorded at 500 MHz with a Bruker AVANCE II 500 spectrometer at room temperature using deuterated chloroform as internal solvent and $CDCl_3$ for ^{13}C with tetramethylsilane (TMS) as reference.

FTIR Spectroscopy

2 mg of purified PHB granules as obtained above were removed and thoroughly mixed with 100 mg spectroscopic grade KBr in a mortar and pestle. From this mixture, 15mg was used for making

KBr pellets. The pellets were kept in an oven at 100°C for 4h to remove atmospheric moisture from the sample. FTIR spectrum was recorded on ThermoNicolet IR 200 spectrophotometer. The samples were scanned between 400 and 4000 wave number (cm^{-1}). PHB obtained from Sigma Chemicals was considered as standard.

Preparation of derivatives for GC – MS analyses

A portion of the purified PHB granules as obtained above was dissolved in 2ml of acidified methanol containing 3% (v/v) H_2SO_4, and 1ml of chloroform in a screw-capped test tube. The samples were then kept at 100°C for 60 min. After cooling to ambient temperature, 1ml distilled water was added and shaken for 10 min, after which the two phases were allowed to separate. The organic phase was analyzed with the help of GC (Shimadzu 2010) and the mass spectra were further elucidated using MS (Shimadzu QP2010 Plus)[19].

DSC

To investigate the morphological state of PHB granules, the melting temperature and the enthalpy of fusion of purified PHB granules were measured with DSC (DSC 822^e Mettler toledo).

TGA

The thermal stability of native PHB granules was examined with thermogravimetric analyzer (TGA) (TGA/SBTA 851^e Mettler toledo) operated at nitrogen flow rate of 20 ml/min and a scanning rate of 10°C/min.

RESULTS AND DISCUSSION

Isolation and Identification of the strains

In our earlier studies thirteen haloarchaeal isolates that had been isolated from salt pans around Bhavnagar, Gujarat coast and that exhibited different morphological characteristics had been screened for PHB production. Isolate NPW – 9 that exhibited maximum PHB accumulation was selected for further studies. Based on biochemical analyses (data not shown) [15] the isolate was identified as *Haloarcula* sp.

Phylogenetic analysis

Results of 16S rDNA partial sequencing (642 bp) of the strain NPW – 9 showed maximum sequence identity (100%) with the complete sequence of *Haloarcula* sp. AB19 (GenBank Accession No. DQ471854.1) (Fig. 1). Thus, the isolate is affiliated to *Haloarcula* species and hence in the present study it is referred to as *Haloarcula* sp. AB19.

Characterization of PHB

^{1}H NMR

The ^{1}H NMR scans of the polymer from *Haloarcula* sp. AB19 were recorded. A doublet was recorded at 2.5 ppm for CH_2a and 2.4 ppm for CH_2b, both corresponding to methylene group (- CH_2 -), while a multiplet signal at 5.2 ppm corresponded to the methyne group (- CH -). Another doublet signal at 1.2 ppm corresponded to the methyl group (- CH_3 -). The methyl esters showed a sharp signal around 3.50 ppm, corresponding to the CH_3 – O group of the esters, while the ^{1}H chemical shift referred to $CHCl_3$ was at signal 7.26 ppm[20]. The NMR spectrum obtained is in accordance to the data reported in the literature[21-25]. The chemical shift, multiplicities, and coupling constants of these signals were consistent with their identification as methyl, methylene, and methane protons (Fig. 2).

^{13}C-NMR

The ^{13}C-NMR spectrum showed four main peaks at 169.134 ppm for O-C=O group (carbon belonging to the carboxylic group of butyrate), 67.608 ppm for H-C-CH_3 (carbon of the CHOH group belonging to the butyrate), 40.789 ppm for HC-HC-C=O group (carbon of the methylene group adjacent to the carboxylic group of butyrate) and 19.761 for CH_3 group (carbon of butyrate group), while the ^{13}C – NMR chemical shift referred to $CHCl_3$ was at 77.0 ppm for ^{13}C – NMR. The results obtained are in accordance with data reported in the literature for PHB.

In the fully coupled spectrum, the last three signals were split into a doublet, a triplet, and a quartet, respectively, confirming that they originated from methane, methylene, and methyl carbon atoms; the singlet at 169.03 ppm was attributed to a carbonyl resonance[20, 23, 26] (Fig. 3).

FTIR

The FTIR spectrum of PHB showed presence of intense sharp blend at 1723.57 cm^{-1} of carbonyl stretching (C=O), as supported by data reported in the literature[11, 25, 27, 28]. The blend at 2976.47 cm^{-1} and 2933.30cm^{-1} was alkyl (CH_3) group, bend of –OH– group appeared at 3448.44cm^{-1}. The band at 1461 cm^{-1} is due to asymmetric bending of –CH_3 and at 1281.20 cm^{-1} was due to C-O stretching. The FT IR absorption band at about 1730 cm^{-1} is a

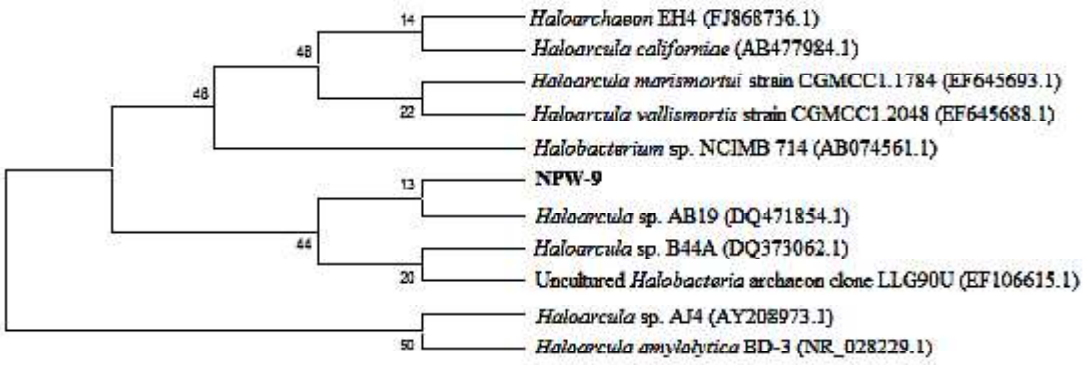

Fig. 1. Phylogenetic tree using neighbor-joining method

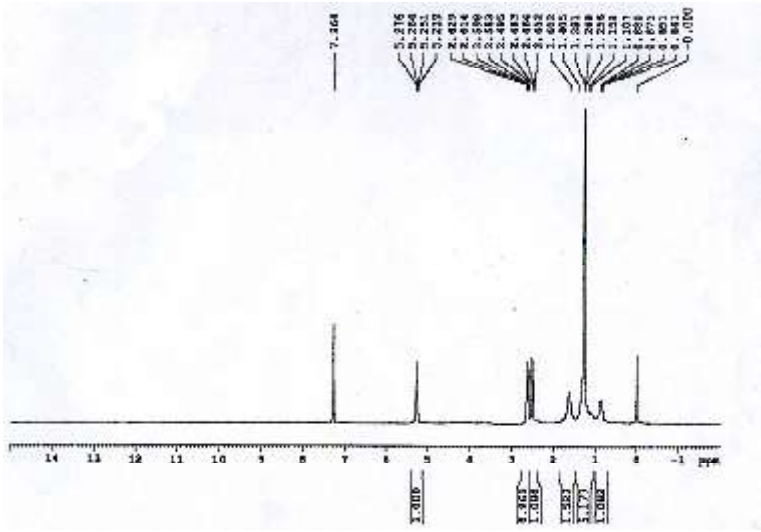

Fig. 2. [1]H NMR of PHB purified from *Haloarcula* sp. 1 AB 19

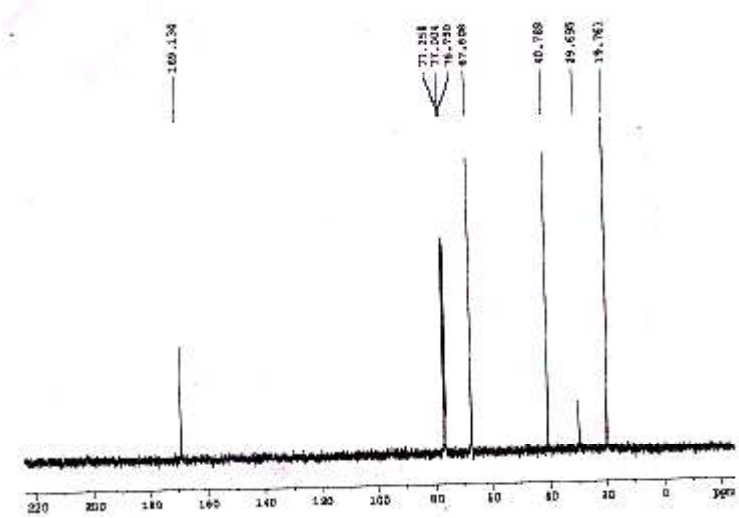

Fig. 3. [13]C NMR of PHB purified from *Haloarcula* sp. 1 AB 19

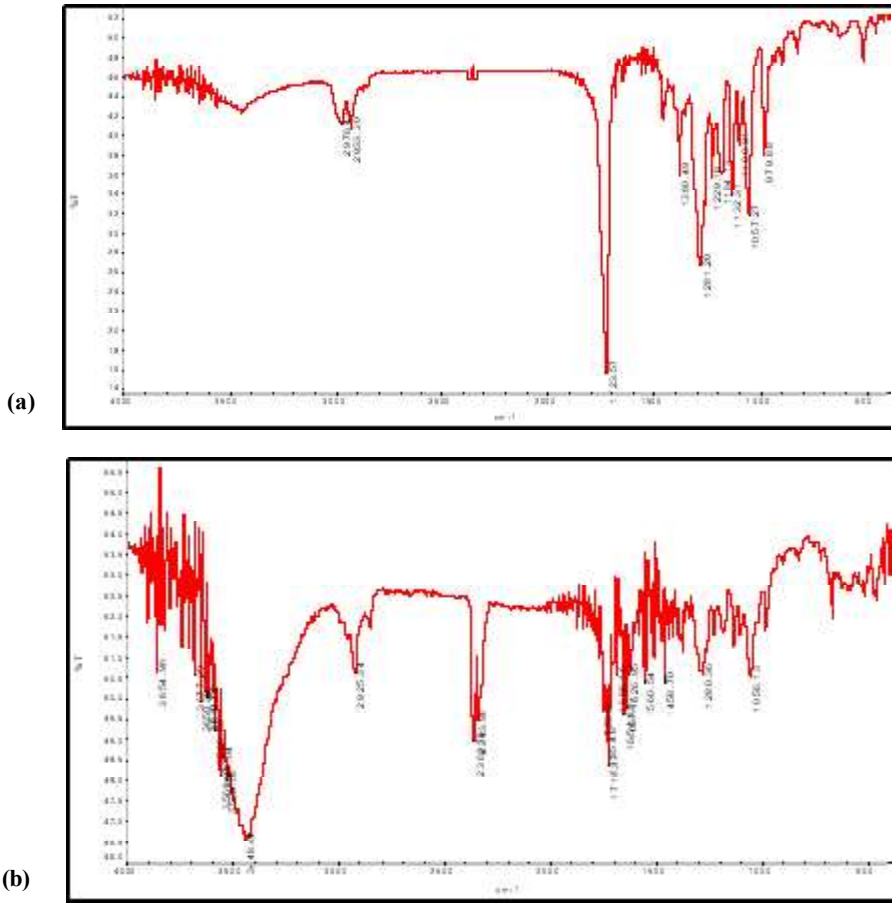

Fig. 4. FTIR of PHB purified from *Haloarcula* sp. 1 AB 19 (a) Standard (b) Sample

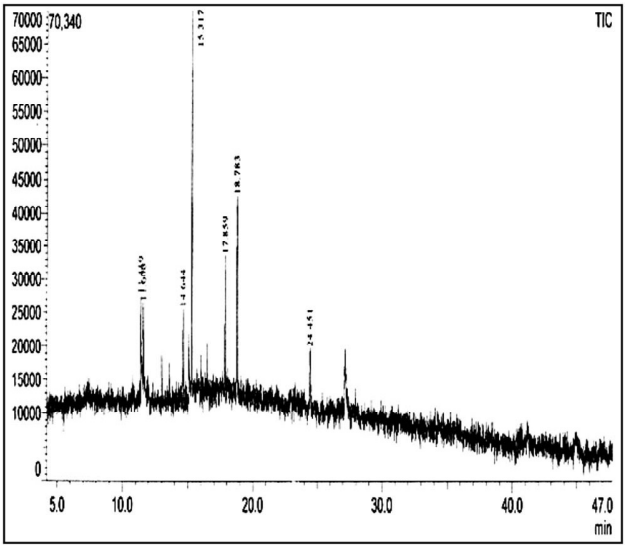

Fig. 5. Gas Chromatographic analysis of PHB from *Haloarcula* sp. 1 AB 19

characteristic of the carbonyl group and that a band at about 1281.02 – 1057.27 cm^{-1} characterizes valance vibration of the carboxyl group[25, 29] (Fig. 4).

GC–MS

GC is commonly used to detect PHB[19]. The peak corresponding to propyl ester of 3 – hydroxybutyric acid was observed in the gas chromatogram, indicating the accumulated polymer to be PHB. Peak at 15.31 is propyl ester of benzoic acid (internal standard) and peak at 12.45 is propyl ester of 3 – hydroxybutyric acid. The peaks at 17.8 and 18.7 that appeared in gas chromatogram may be attributed to the presence of analogues of PHBs, which may be present in the crude extract (Fig. 5).

The methanolysis products of the polymer samples were also analyzed using GC – MS. By analyzing the fragmentation patterns and the molecular mass of the fragments, the specific peaks in the spectra correlated to the carbonyl and

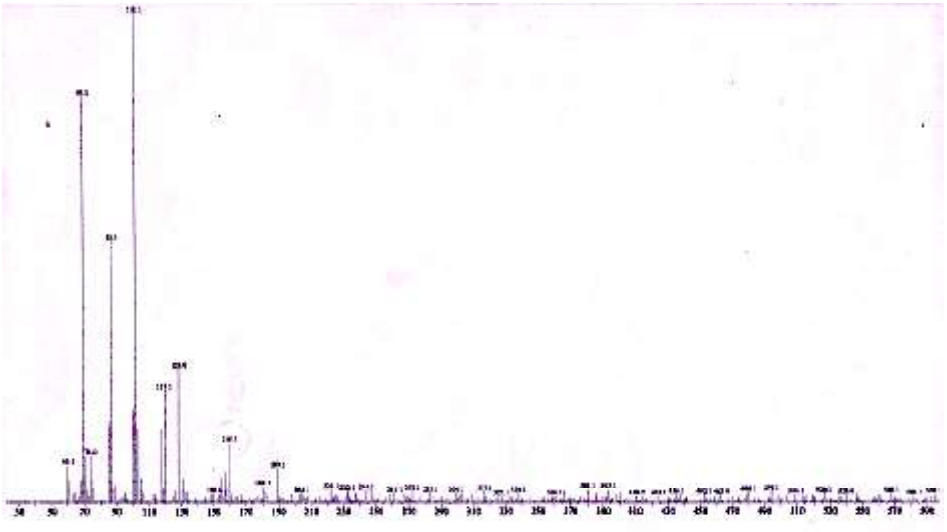

Fig. 6. Gas Chromatography – Mass Spectroscopy of PHB from *Haloarcula* sp. AB 19

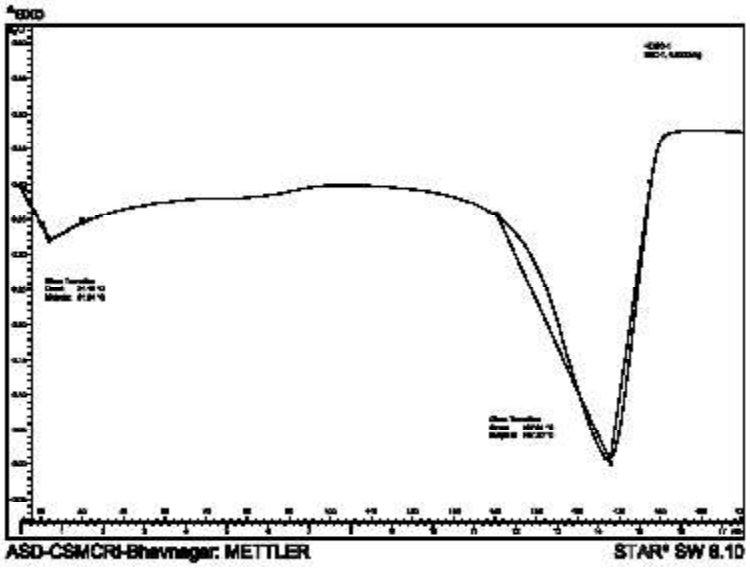

Fig. 7. DSC thermogram of PHB produced from *Haloarcula* sp. AB 19

hydroxyl ends of the representative hydroxyalkanoates. The spectrum exhibited the main diagnostic peaks; one at m/z 101 which is the main fragment due to loss of the water molecule from the molecular ion, peak at m/z 69 represented the ion due to the combined loss of both water and methanol, peak at m/z 87 due to loss of methanol molecule from the protonated molecular ion. The peak at m/z 119 represented protonated molecular ion MH+. The GC-MS results obtained is in accordance to data reported in literature[20, 30] (Fig. 6).

DSC

The calorimetric scan of PHB extracted from the isolate *Haloarcula* sp. AB19 is shown in Fig. 7. The temperature at which the peak is obtained is considered as the temperature of melting (T_m), if two peaks are obtained, the higher value is considered as T_m. The melting temperature of the polymer ranged between 170 and 174 °C[30, 31]. The T_g value obtained for the polymer was found to be 15 °C.

TGA

A degradation temperature for the polymer obtained by TGA curve is shown in Fig. 8. The figure indicated that the polymer underwent single step thermal degradation and was stable up to 260 °C. Thermal degradation was observed in the range of 260 °C to 340 °C. Thermostability of the polymer was upto 230 ºC[32].

Investigations on PHB production by moderate halophiles have been initiated with the studies on *Halomonas boliviensis* LC1[33]. PHB has been reported to be produced by very few haloarchaea, notably species of *Haloferax*, *Haloarcula* and *Halobacterium* only[34]. Hence, the present study gains importance where halophilic Archaea emerge as the organisms of choice due to their unique features as no sterilization procedures are required and thus owing to simple cultivation requirements[18]. A further advantage of PHB production by halophilic Archaea is the ease of recovery of the polymer by hyposmotic shock of the cells on treatment with salt deficient water, hence reducing the downstream processing costs. They also have versatility in the choice of a broad range of substrates and simple carbon sources such as sugars, acetate or succinate that favors the yield of PHB and growth directly reducing production cost[35, 36, 37]. This is one of the rare reports on exploitation of haloarchaea for the production of PHB and its complete chemical characterization.

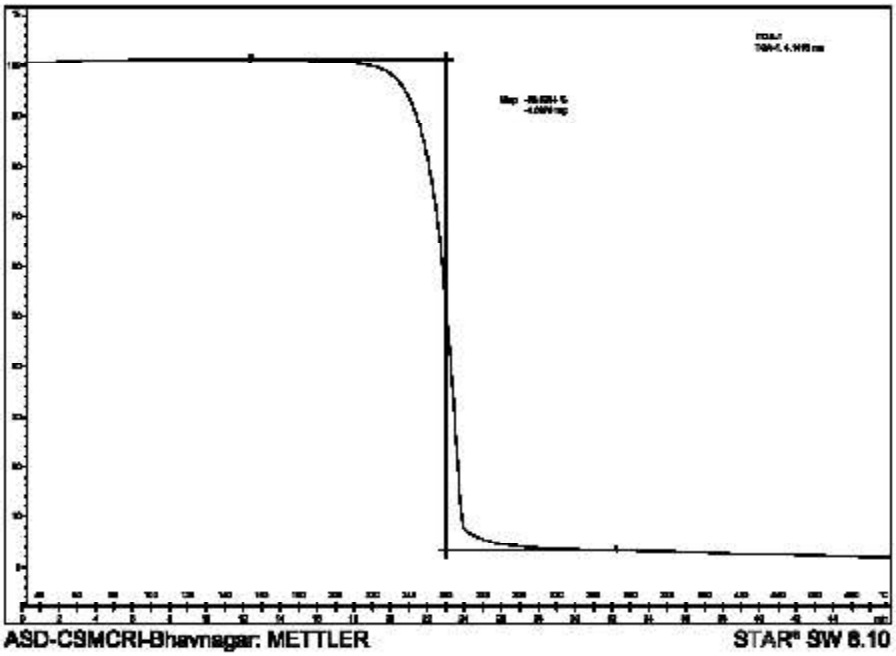

Fig. 8. TGA analysis of PHB produced from *Haloarcula* sp. AB 19

CONCLUSION

To conclude, haloarchaea are an important source of biopolymer. Research on engineering of media components to reduce the time of production is in progress. Further research is needed to improve the mechanical properties of the polymer that hold promise for providing an economically competitive industrial scale production.

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