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Production and characterization of thermostable polyhydroxybutyrate from *Bacillus cereus* PW3A

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Abstract

Polyhydroxyalkanoates are biodegradable, linear polyesters produced primarily by bacteria. Present study reports production and characterization of polyhydroxybutyrate from *Bacillus cereus* isolated from polluted water sample. The study investigated effect of carbon & nitrogen sources and process parameters like incubation time, pH and temperature. The polyhydroxybutyrate was characterized by Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), X-ray Crystallography (XRD) and Nuclear Magnetic Resonance (NMR) studies. Diffraction Scanning Colorimetry (DSC) and Thermo gravimetric Analysis (TGA) provided thermal properties of the polymer. The study demonstrated the thermal stability of the polymer compared to the commercially available PHB suggesting its unique application.

Keywords: Polyhydroxybutyrate, *Bacillus cereus*, carbon and nitrogen sources, process parameters, polymer characterization, thermal stability.

Introduction

Polyhydroxyalkanoate (PHA) is an important biopolymer of microbial origin. It is accumulated in the cells as intracellular granules in the presence of excess carbon source and limited nitrogen source (Anderson and Dawes 1990). The PHA are biodegradable and possess material properties similar to those of synthetic polymers. Poly 3-hydroxybutyrate (PHB) is an important class of PHA which is water insoluble, resistant to ultraviolet radiation, impermeable to oxygen, biodegradable and biocompatible. These properties make PHB an attractive material which has several applications in medicine, food packaging, drug delivery, tissue engineering, etc (Wang and Yu 2007). Both gram positive and gram negative bacteria were earlier reported to produce PHA (Thirumala et al 2010). Gram negative bacteria are reported to produce quantity of PHA suitable for commercial production. However, these gram negative bacterial cell walls possess lipopolysaccharides (LPS) as cell wall constituents which may limit PHA from these sources for biomedical applications.

The genus *Bacillus* is gram positive and seems to be good candidate for the production of PHA/PHB for biomedical applications. Such gram positive bacteria lack the LPS and intensive investigations are required for their exploitation in PHA production. The PHA content and its composition are influenced mainly by the strain of the microorganism, the type of substrate employed, substrate concentration and growth conditions. To achieve a cost effective PHA production, the availability of an efficient bacterial strain is a prerequisite, and is a focus of interest for many investigations (Valappil et al 2007).

Recent developments in biopolymer technology focus towards development of green biocomposites which are biodegradable, regenerable, with reduced density and cost (Ahankari et al 2011). Many studies were also conducted to evaluate sustainability of bio-based plastics. Currently bio-based plastics are used in fibers, coatings, packaging, apparel, toys, inks, food containers, tableware and cutlery (Chavez et al 2012). In the present investigation, PHB accumulating *Bacillus cereus* was isolated and identified. An attempt was also made to produce effective amount of PHA using different carbon and nitrogen sources in mineral salt medium. The PHB produced was characterized using different techniques to suggest suitable application for the same in either pure or composite form.

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Materials and Methods

Isolation and screening

The PHA accumulating organism *Bacillus cereus* was isolated from polluted water sample collected from a treatment plant at Hubballi. The organism was sub-cultured and preserved on nutrient agar slants at 4^{o}C till further use. Sudan black stain (Sudan black B powder 0.3g, 70% ethyl alcohol 100 ml) was used to show microbial intracellular lipid, PHB. PHB granules

accumulated in the organisms were seen as a black spot under oil immersion objective.

Identification of the microorganism

Morphological and biochemical studies were carried out for the isolate. Molecular identification of the organism was done by 16S rRNA sequence analysis. Genomic DNA from the isolate was extracted as per standard protocol described earlier (Sambrook et al 1989). The DNA was stored at -20^{o}C for further use. The DNA isolated from the organism was subjected to polymerase chain reaction (PCR) amplification using Eppendorf thermal cycler. Gel electrophoresis was performed using 1.0% agarose to analyze the size of amplified PCR product. The size obtained was approximately 850 bp for 16S rRNA region. It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained. For sequencing of PCR product 9F-5' GAGTTTGATCCTGGCTCAG 3' sequencing primer was used. The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of the isolate.

PHB production and quantification

The production medium used was a modified mineral salts medium which contained following ingredients: 0.05% KH_2PO_4, 0.02% $MgSO_4$, 0.01% NaCl, 0.25% peptone, 0.25% yeast extract supplemented with 5% carbon source with pH 7.0 (Ramsay et al 1990). For PHB production, 100 ml of production medium was inoculated with the organism at an inoculum level of 2%. All the flaks were incubated at 35^{o}C for 48 h. The samples were centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellets were collected. The pellet was treated with sodium hypochlorite for cell lysis and PHB extracted using hot chloroform. The PHB quantification was done following our earlier study by adding sulphuric acid to PHB and converting it to crotonic acid. Absorbance was read at 235 nm against a sulphuric acid blank (Hungund et al 2013).

Effect of media components and process parameters.

The effect of various parameters was studied to achieve higher PHB yield. To analyze the effect of incubation time, the inoculated flasks were kept at 35^{o}C, pH 7 and 150 rpm for 5 days (24, 48, 72, 96 and 120 hours). For analyzing effect of temperature, the incubation temperature was maintained at 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C. Similarly for analyzing effect of pH, media with different pH values (5, 6, 7, 8 and 9) were inoculated with test organism. Effect of various carbon sources such as glucose, fructose, sucrose and galactose were studied. Nitrogen sources like beef extract, peptone, tryptone and yeast extract affecting PHB production was investigated.

Characterization of the produced polymer

Morphological characteristics of the sample were examined using Scanning Electron Microscopic technique at an operating voltage of 20 kV using a secondary electron detector on JEOL JSM - 6390LV. Characteristic X rays from the sample were detected using Energy Dispersive Spectroscopy technique performed on JEOL JED – 2300 to obtain the composition of the sample. For FTIR study, 2 mg of sample was thoroughly mixed with KBr and 15 mg treated pellet was dried at 100^{o}C for 4 h. FTIR spectrum was acquired using NICOLET 6700 (USA) Fourier Transform IR Spectrometer. The enthalpy of fusion and melting temperature was measured using Differential Scanning Calorimeter from SDT Q 600 TA Instruments, USA and Q 20 TA Instruments, USA. Samples of 15-20 mg were encapsulated in aluminum pans and heated from 0 to 200^{o}C at the rate of 20^{o}C/min. The melting temperature was taken at the peak of the melting endotherm. Thermogravimetric analysis of PHB sample was performed with 10 mg sample with 200 ml/min nitrogen gas flow rate on Perkin Elmer, Diamond TGA equipment. X-ray diffraction experiments were performed on Bruker AXS D8 Advance Diffractometer with X ray (Cu Kα (λ = 0.1506 nm) beam operated at 40 kV and 35 mA to study the structural properties of the sample. Si (Li) PSD detector was used to record the data in 2θ range of 6^{o} to 80^{o}. The ^{1}H NMR spectra were acquired with a Bruker Avance III, 400 MHz spectrometer to study the structural elucidation. Known quantity (5 mg) of the extracted polymer samples were dissolved in $CDCl_3$ for this experiment.

Results and Discussion

Microorganism

Morphologically, the isolated strain appeared as off-white, circular, smooth, large colony. Microscopically it appeared as rod shaped gram positive spore forming bacillus. Growth was observed over a wide range of temperatures (15^{o}C-45^{o}C). Identification of the strain was based on 16S rDNA sequencing (GenBank accession number KT808623) and identified as *Bacillus cereus* (PW 3A). Microscopic observation performed on cells during the accumulation of PHB clearly showed the existence of intracellular PHB granules. The presence of endospores and the positive test for catalase activity for the organism indicated that the isolate could be a *Bacillus* species. The isolated PHB producing bacteria were shown to accumulate PHB via microscopic staining techniques using Sudan black B stain. The isolate displayed reactivity in the form of intense purple or black granules/cells against safranin counter-stained background. The molecular phylogeny of sample was determined by analyzing 16S rRNA gene sequences. On the basis of position of sequence of the given bacterial sample in the phylogenetic tree, the isolate showed closest similarity with *Bacillus cereus* (Figure 1). Earlier such studies also reported isolation and characterization of PHB from *Bacillus* spp. isolated from natural habitats (Thirumala et al 2010; Aarthi and Ramana 2011).

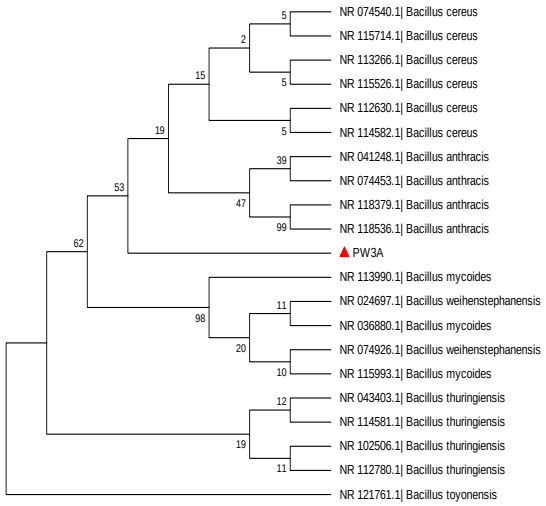

Figure 1: Phylogenetic tree for *Bacillus cereus*

Effect of media ingredients and growth conditions

Extracting the PHB using the earlier protocol, after evaporation of the chloroform, PHB was observed to be depositing as thin film (Figure 2). Production of PHB was seen at early 24 h of incubation. The highest production of PHB was after 48 h of incubation. Correspondingly there was decrease in glucose consumption in the course of time. Also the concentration of PHB decreased after 48 h (Figure 3). This reduction in PHB production after 72 h may be due to lack of micronutrients as well as increase in metabolites that might have negative effect on the PHB production. The effect of different carbon sources like glucose, fructose, sucrose and galactose on PHB production was studied after 48 h of incubation. The study of effect of carbon sources gave PHB yield of 0.325 g/L, 0.317 g/L, 0.298 g/L and 0.264 g/L respectively when glucose, fructose, sucrose and galactose were provided. The results showed that the organism produced almost same amount of PHB when glucose and fructose were provided as carbon sources. However, PHB yield was moderate when sucrose was provided in the medium and galactose has lowest concentration of PHB (Figure 4).

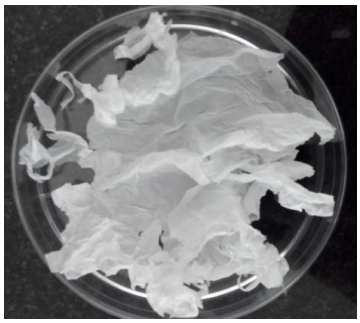

Figure 2: PHB produced by *Bacillus cereus*

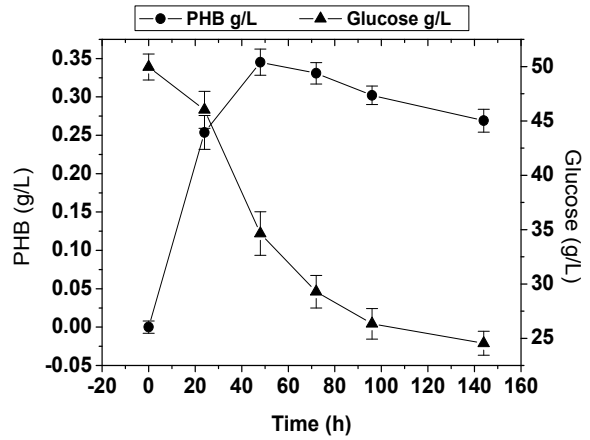

Figure 3: Profile of residual glucose and PHB yield from *Bacillus cereus*

The superiority of the glucose in increasing PHB is in line with study by Choi and co-workers (Choi et al 1995). Working with different carbon sources in MSM broth, Khanna and Srivastav (2005) observed higher PHB yield on fructose by *Ralstonia eutropha.* They reported that glucose and fructose, being monosaccharide were readily utilized by bacteria and, hence, have supported growth and subsequently PHB production. The effect of different organic nitrogen sources like peptone, beef extract, yeast extract and tryptone on PHB production was studied after 48 h of incubation. The study of effect of nitrogen sources, gave PHB yield of 0.3078 g/L, 0.3309 g/L, 0.2801 g/L and 0.3284 g/L respectively when beef extract, peptone, tryptone and yeast extract were provided. Peptone and yeast extract gave high concentration of PHB while tryptone has the least (Figure 4). Page (1992) tested PHB production in a variety of commercially available complex nitrogen sources (fish peptone, protease peptone, yeast extract, casitone, phytone and tryptone). It was found that complex nitrogen sources increased the yield of PHB produced by *Azotobacter vinelandii* UWD strain. Similar results were obtained from cultivation of *Bacillus licheniformis, Anaerobiospirillum succiniproducens* and *Phafia rhodozyma* in the presence of yeast extract, and a combination of yeast extract and peptone (Lee and Choi 1999). The studies here revealed that PHB is produced with wide range of pH 6-9 (Figure 5). Neutral pH around 7.0 is the most favourable pH for bacterial growth and hence, has contributed to higher PHB production. PHB being a growth associated product is produced in the temperature range of 25°C to 45°C and the highest PHB production was observed at 35°C (Figure 5). The above results are also supported by various studies by other research groups.

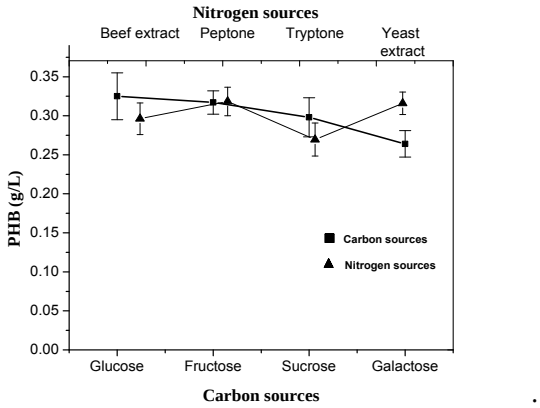

Figure 4: Effect of carbon and nitrogen sources on PHB production by *Bacillus cereus*

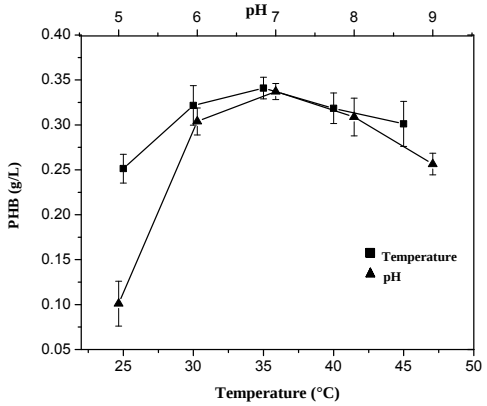

Figure 5: Effect of temperature and pH on PHB production by *Bacillus cereus*

Characterization of the polymer

Images obtained from scanning electron micrographs reveal the presence of pores of different size throughout the sample as shown in Figure 6. The surface consists of multiple pores with different sizes and the average pore size was found to be 24.4071 µm which is in accordance with the reported studies (Raouf et al 2006). The EDS spectrum shows the presence of

carbon at 0.277 keV with 93.13% mass percentage and oxygen at 0.525 keV with 6.87% mass percentage (Figure 7). No other peaks were observed in the EDS spectra which rules out the presence of any other elements in PHB sample. This indicates that PHB produced is in the pure form. FTIR spectrum (Figure 8) obtained for the extracted polymer shows peaks at 1726.33 cm^{-1} and 1283.58 cm^{-1} which corresponds to specific rotations around carbon atoms specific to certain functional groups. The peak at 1726.33 cm^{-1} corresponds to C=O (carbonyl) stretch of the ester group present in the molecule. The peak at 1283.58cm^{-1} corresponds to –CH group. Absorption bands occurring at 2924.61 cm^{-1} and 2863.78 cm^{-1} indicate the presence of aliphatic $-CH_3$ and $-CH_2$ groups. These peaks are similar to that obtained for the standard PHB (Make: Sigma) at 1728 cm^{-1} and 1282 cm^{-1} confirming that the extracted polymer is PHB [19]. The results are also in agreement with the studies of other research groups working on PHB extracted from *Bacillus cereus* and *Bacillus mycoides* and *Bacillus thuringiensis* (Rohini et al 2006).

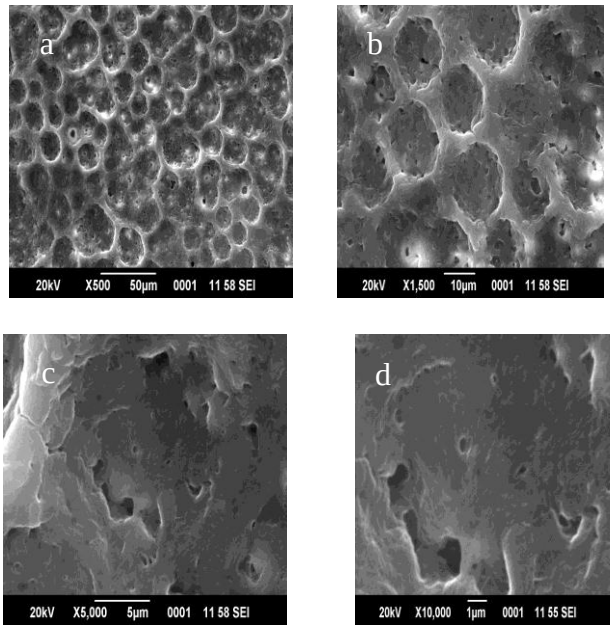

Figure 6: Scanning electron micrographs of the PHB surfaces at 20 kV with magnification of: a) 500 X; b) 1500 X; c) 5000 X and d) 10000X.

The DSC spectrum (Figure 9) shows a peak at 165.57°C representing melting point of PHB and the corresponding crystallization temperature at 159.14°C. The enthalpy of fusion was 79.26 J/g and crystallinity during melting in the first cycle was calculated to be around 60-65% assuming the enthalpy of fusion of 100% crystalline sample to be 146 J/g. The observed melting temperature in this study was in accordance with that reported for *Bacillus thuringiensis* i.e. 165.6°C and the enthalpy of fusion was found to be 84.1 J/g (Sindhu et al 2011). According to the literature, commercial PHB has melting temperature around 170°C – 180°C (Rohini et al 2006). The thermal degradation of extracted PHB as revealed by Thermogravimetric analysis proceeds by a two-step process with decomposition temperatures at 214.9°C and 413.3°C (Figure 10). This thermal degradation at decomposition temperature of approximately 300°C is mainly associated with the ester cleavage of PHB component by β-elimination reaction (Chaijamrus et al 2008). The residual mass percentage was calculated to be 23.16% and 3.1% after the first and second steps respectively. The decomposition temperature for standard PHB from Sigma Aldrich is 302°C (Sindhu et al 2011). Higher decomposition temperature in our case shows that our PHB sample exhibits better thermal stability. This may be due the presence of impurities such as proteins and lipoproteins from the non-extracted cell mass (Choi and Lee 1997).

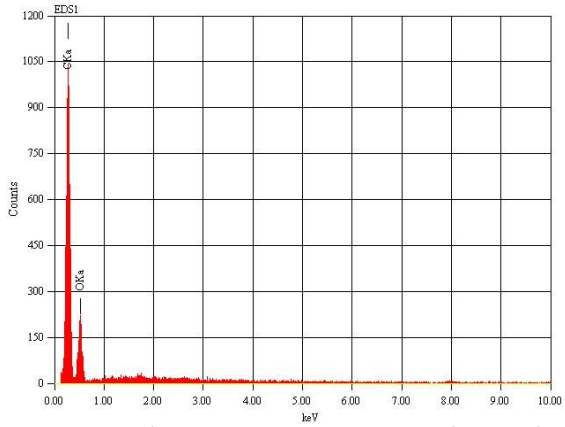

Figure 7: EDS profile showing elemental composition of the PHB from *Bacillus cereus*

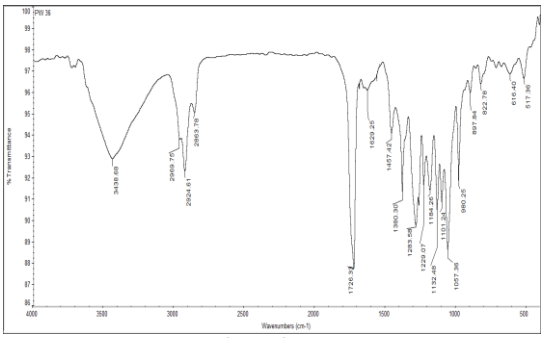

Figure 8: FTIR spectrum of PHB from *Bacillus cereus*

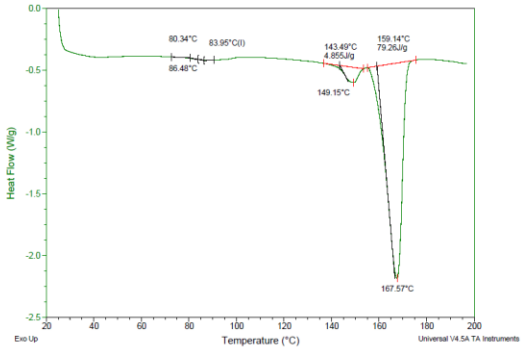

Figure 9: DSC profile of PHB from *Bacillus cereus*

The XRD pattern of PHB extracted from *Bacillus cereus* shows characteristic peaks at 2θ values of 13.462, 16.819, 25.388, 26.894, 35.573 and 53.876 respectively (Figure 11). These values are similar to that of standard PHB having 2θ values of 12.1, 13.4, 15.2 , 16.8, 20.0, 22.4 and 25.4 (Rooy et al 2007). All these peaks possess narrow FWHM which signify the high crystalline quality of the PHB produced. The higher intensities of these peaks indicate higher order of crystallinity of the PHB. Broader peaks present in the XRD spectrum do not belong to PHB and are due to the impurities, which might have been incorporated unintentionally while handling the specimen during experimentation and characterization. The

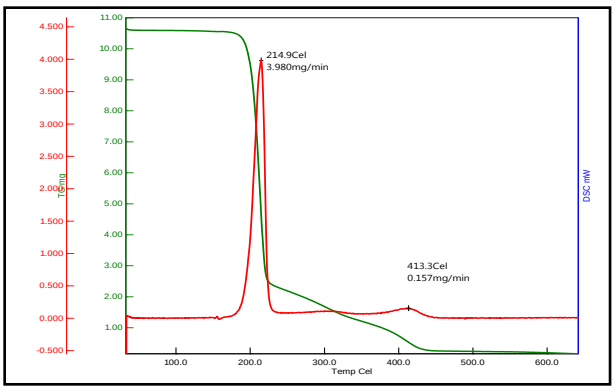

Figure 10: TGA profile of PHB from *Bacillus cereus*

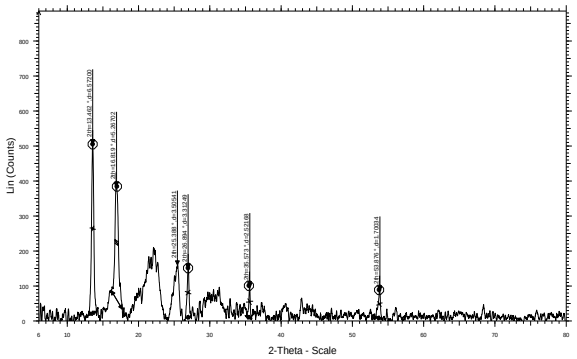

Figure 11: XRD spectrum of PHB from *Bacillus cereus*

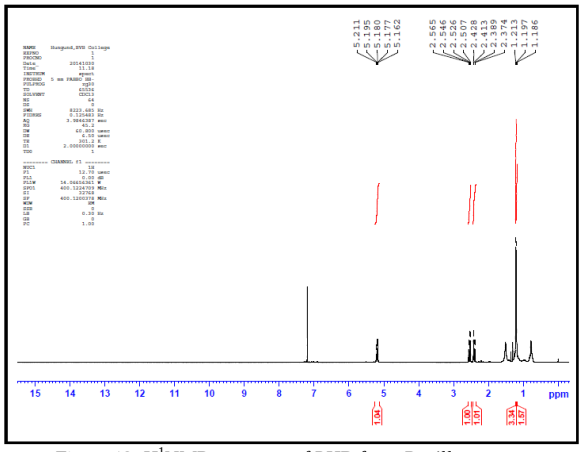

Figure 12: H^1NMR spectrum of PHB from *Bacillus cereus*

H_1 NMR spectrum for *Bacillus cereus* is compared with that of commercial PHB by Sigma Aldrich, USA and it is noted that they match each other perfectly. The peaks observed in the spectra coincide, corresponding to the different types of carbon atoms presented in the PHB structure, $[-O - CH - (CH)-CH - (C = O) -]_n$. Detailed observation of the spectrum shows the presence of three groups of signals characteristic of PHB homopolymer, triplet at 1.21 ppm attributed to methyl group coupled to one proton; the doublet of the quadruplet at 2.565 ppm to methylene group adjacent to asymmetric carbon atom bearing a single proton and the multiplet at 5.211ppm to the methyne group (Figure 12). Two more signals at 1.5 ppm and 7.2 ppm correspond to the solvent, chloroform. The NMR signal multiplicity by a proton as an octet in case of proton of CH_2 group was obtained due to proton coupling in isotactic form (Chaijamrus et al 2008; Shalin et al 2013).

Conclusion

Among the several biodegradable polymers, polyhydroxyalkanoates are considered as suitable biodegradable thermoplastics for several applications. PHB production using renewable carbon sources attracts much importance. In this study, the isolated culture *Bacillus cereus* has produced PHB having concentration of 0.3453 g/L. The characterization of the extracted polymer has proved it to be more thermostable compared to the commercially available polyhydroxybutyrate. Further studies are required to blend the extracted polymer with suitable nanoparticles to improve its thermal and mechanical properties for future applications.

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