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Characterisation of Polyhydroxy Butyrate (PHB) from *Bacillus subtilis* Strain P Isolated From Rhizosphere Soil

Jini Joseph* and O. S. Chithira

Department of Biotechnology, St.Peter's College, Kolenchery, Kerala, India

**Corresponding author*

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A B S T R A C T

Polyhydroxy Butyrate (PHB) is a biodegradable polymer material with nontoxic characteristics. In this paper we report PHB production from bacterial isolate from rhizosphere soil. PHB production by the isolates was confirmed by Sudan black and Nile blue staining methods. The physical, colonial, morphological and biochemical characteristics of the isolate were noted. For further confirmation of identity, 16s rDNA sequencing and BLAST search was performed. The results showed that the isolate had highest homology (100%) with *Bacillus subtilis* strain *P*. The phylogenetic tree was constructed to identify the evolutionary relatives of the isolated bacteria. The optimum conditions for PHB production were studied. An optimum PH of 7and incubation period of 24 hours were found to be appropriate for maximum production of PHB. Yeast extract and Mannitol were found to be most ideal nitrogen and carbon source respectively for the production of PHB. The extraction of PHB was done using crotonic acid method and further the characterization was done by FTIR and NMR analysis. The peaks in the FTIR data corresponds to the probable presence of C=O valence, CH_2-S groups or ester C-O groups whereas the molecular composition of the polymer as indicated NMR analysis, indicates the presence of CH bridges.

Introduction

Plastic materials have become an integral part in our life as a basic need but they are causing serious environmental problems due to their non-biodegradability. Synthetic polymers obtained from petrochemicals causes air pollution only because they are not degradable in soil for long time. Extensive use of petroleum-derived plastics (approximately 269 million tons used globally in 2015) increases the environmental concerns of non-biodegradable wastes, including contamination with small fragments of toxic compounds leaching out of landfills into ground water and the emission of greenhouse gases and other organic pollutants during the degradation process (Bernard, 2014).

Biodegradable biopolymers (BDP) are an alternative way for the production of petroleum-based polymers like, traditional plastics. Some BDP degrade in only a few weeks, while the others take several months

for the degradation. The BDP can be produced from natural raw materials such as starch, sugar, cellulose as well as fossil oils. Besides the biodegradability, other relevant aspects of biopolymers including thermal stability and viscosity can be achieved by conventional technology without any large adaptations. The BDP also protects the loss of soil fertility and significantly enhance soil carbon level (Ahmad *et al.,* 2017). Polyhydroxyalkanoates (PHAs) are a group of polyesters synthesized by bacteria, archaea as well as some fungi (Weiner, 1997; Kim and Lenz, 2001; Rehm, 2010) These polyesters are biodegradable and biocompatible. The PHA types are polyhydroxybutyrate (PHB), Polyhydroxyvalerate (PHV), polydroxyhexanote (PHH) and polyhydroxy-octanoate (PHO). Polyhydroxy alkonates (PHA) are polyesters of hydroxyl-alkanoates (HA) and consists of betahydroxyacyl as monomer (Swathy and SruthyRanjini, 2015). Among the PHAs, poly(3-hydroxybutyrate) [P(3HB), PHB] is the best characterized PHA (Penaetal., 2014).The properties of PHAs are also similar to those of polyethylene (PE) and polypropylene (PP).

At least 75 different genera of bacteria have been known to accumulate PHB as intracellular granules. Its production has most commonly been studied on microorganisms belonging to the genera *Alcaligenes sps., Azotobacter sps., Bacillus sps.,* and *Pseudomonas sps* (Shamala *et al.,* 2003). PHB is primarily a product of carbon assimilation and is used by micro-organisms as a form of energy storage molecule.

It can be made into many forms and shapes. There are currently a variety of methods for detecting and identifying microorganisms that synthesize and accumulate intracellular PHAs granules (Spikermann *et al.,* 1999; Sheu *et al.,* 2005; Solaiman *et al.,* 2000)

Materials and Methods

Sample collection

Soil samples were collected from 10 different sites in and around Ernakulam District, Kerala and transported in sterile bags to the Microbiology Laboratory of St. Peter's College, Kolenchery for further bacterial isolation.

Isolation and primary screening of PHB producing bacteria

The central portion of the collected samples were aseptically transferred to sterile polythene bags and carried to the laboratory and maintained at 4^0C for further study. The collected sediment samples were serially diluted and spread plated on Yeast Mannitol Agar (YMA) medium and incubated for 24 hrs at 37^0C. The appearance of the colonies on YMA medium is an indication for PHB production. The single colonies were re-inoculated onto fresh agar slants.

Conformation of PHB producing bacteria

Sudan black staining

Sudan black is non-fluorescent lysochromediazo dye used for staining of neutral triglycerides and lipids. The presence of PHA as intracellular granules was confirmed by staining the cells with Sudan black-B. Several drops of microbial broth were fixed on a glass slide by applying heat, then it is stained with 3% Sudan Black B in 70% ethanol for 10 minutes. The slide was then immersed in xylene until completely decolonized. Then the sample was counterstained with safranin (5% w in D.W) for 10 seconds. The slides were washed with distilled water, dried and examined by optical microscopy (Burdon *et al.,* 142)

Nile blue staining

Nile blue sulfate is a basic oxazine dye and it is used to stain the intracellular PHA granules. For this, 0.1% of Nile Blue A dye is added to YMA agar medium and kept for overnight. The colonies that produce PHB emit fluorescence (Lakshmanan, 2004).

Morphological characteristics

The morphological identification of isolate was conducted. Colonies were compared for their diameters, overall colours, texture, size, cell arrangement, elevation, pigmentation and optical property. The morphological identification of the strain was done according to the method of Cappucino and Sherman (2007)

Molecular identification of bacteria

Polymerase chain reaction allows amplification of specific DNA sequences. Pure culture of the target Bacteria was grown overnight on nutrient broth for the isolation of DNA. The DNA was isolated from the bacteria using phenol-chloroform method (Hoffman and Winston, 1987) and 16S rDNA was amplified by Thermocycler (Eppendorff) using the universal primers, 27F and 1492R (Lane,1991) (Forward primer: 5'AGAGTT TGATCCGGCTCAG3";Reverseprimer:5'AC GGCTACCTTGTTACGACTT-3' ') The PCR was performed in 50µl reaction mixture containing 5µl of 10X assay buffer, 4µl dNTP mix, 1µl each of forward and reverse primer (100µmol), 0.3µl of Taq polymerase, 5µl of template DNA and 33.7µl of HPLC grade water.

The amplified 16S rDNA PCR product was sequenced using an automated sequencer (SciGenom Labs Pvt Ltd, Kochi, India.). The Sequence Similarity search was done for the 16S rDNA sequence using an online search tool called BLAST. The unknown organism was identified using the maximum aligned 16SrRNA sequences available in the GenBank of NCBI through the BLAST search. The best sequence alignment results were noted.

Phylogenetic Tree

The 16S rRNA sequence of microbial isolate PHB 5 was analysed using the BLAST tool in NCBI. Multiple Sequence Alignment was performed using MEGA 6 software. On the basis of these results a phylogenetic tree was constructed which indicated the evolutionary relationship of *Bacillus subtilis* strain P with closely related species.

Extraction of PHB

Chloroform method

The bacterial cultures were harvested by centrifugation at 5000 rpm for 10 min. The lipids were removed from the cell pellet-using methanol (40 times the volume of cell pellets) and the cells were incubated at 95°C for 1 h. Then it was filtered to remove methanol completely and the sediment granules were incubated in an oven at 65°C till dry. Chloroform was added to the dried granules and was incubated at 95°C for 10 min and after cooling, the mixture was gently mixed overnight. The solution was then filtered to get the debris. Finally, the PHA was precipitated from the debris with 7:3 (v/v) mixtures of methanol and water. The precipitated PHA was then washed with acetone and dried (Santhanan and Sasidharan, 2010).

Quantification of PHB

PHB crystals were converted into crotonic acid by adding sulfuric acid at 100°C in water bath.The absorbance of the solution was

measured at 235 nm in a UV-visible spectrophotometer against a sulfuric acid used as a blank. The amount of PHB per gram dry weight of bacterial cells was determined using a standard curve of PHB. Quantification of PHB was done by crotonic acid method (Panigrahi and Badveli, 2013). Percentage yield was found to be 0.3 g.

Fourier Transform Infrared Spectroscopy (FTIR)

A small quantity of the sample was added to KBr in the ratio 1:100 approximately. The matrix was ground for 3-4 minutes using mortar and pestle. The fine powder was transferred into a 13 mm diameter die and made into a pellet using a hydraulic press by applying a pressure of 7 tonnes. The fine pellet was subjected to FTIR analysis using universal pellet holder. (a single drop of oil is poured on the KBr pellet in case of liquid samples). Infrared spectral data were collected on Thermo Avtar 370 FTIR spectrometer. Spectra are collected over a range of 4000–400 cm−1at 4 cm−1 resolution with an interferogram of 32scans.

Nuclear Magnetic Resonance Spectroscopy (NMR)

The samples were dissolved in denatured chloroform (CDCl3) and the solution was analysed at 400 MHz on an AMX 400 (Bruker) spectrophotometer. The spectrum was recorded at 25°C with pulse repetition of 3s (Lakshmi, 2004).

Results and Discussion

Isolation and screening of PHB producing bacteria

The well grown bacterial culture isolated from the rhizosphere soil near Kolenchery, Ernakulam District, Kerala was used for screening for PHB production using Sudan Black and Nile Blue staining methods. The bacterial isolates present in the samples were isolated by serial dilution (spread plate) technique. After incubation, morphologically distinct bacterial colonies were isolated and purified from the plates. The well grown bacterial cultures labelled as PHB5 was used for further screening techniques.

Secondary screening

Sudan Black Staining

Sudan black is nonfluorescent lysochromediazo dye used for staining of neutral triglycerides and lipids. Sudanblack stained the PHB granules as dark blue, while the cells appeared in pink colour(Fig.1). Colonies unable to incorporate the Sudan black B appeared white, while PHB producers appeared bluish black (Juan *et al.,* 1998). Further screening of PHB producing bacteria were done by a more rapid and sensitive, Nile blue staining method.

Nile blue staining

Nile blue sulfate is a basic oxazine dye and it is used to stain the intracellular PHA granules. The PHB producing colonies in the plate emitted blue coloured fluorescence. (Fig 2)

Morphological, cultural and biochemical characteristics

The isolated PHB producing bacterial colonies were characterized for their morphological and biochemical characteristics as described by Cappuccino and Sherman (2007). The strain labelled as PHB 5 was found to be gram positive. Furthermore, the biochemical analysis of the isolates was performed and identified to be *Bacilli* species. Their colonial, morphological,

and biochemical characteristics are tabulated in Table 1 and Table 2

Optimization of culture condition for maximum PHB production

The optimization of fermentation conditions, particularly physical and chemical parameters are of primary importance in the development of any fermentation process owing to their impact on the economy and practicability of the process. Several cultural parameters were evaluated to determine their effect PHB production. Growth conditions including pH, temperature, carbon and nitrogen sources play an important role in the production rate of PHA.

In the present study pH 7 was found to be the optimum for maximum PHB production. The other parameters most favourable for PHB production were found to be an incubation period of 24 hours, yeast as the nitrogen source as well as mannitol as the carbon source for submerged fermentation. Similar results were obtained from cultivation of *Bacillus licheniformis, Anaerobiospirillum Succiniciproducens* and *Phafiarhodozyma* (Lee *et al.,* 2000; Shah, 2014)

Effect of pH on PHB production

Maximum PHB production was obtained at pH 7 (10µg/ml) at 37°C. The PHB production was found to decrease when pH was increased from 8 to 9 (Table 3).

Effect of incubation period on PHB production

Maximum PHB production was obtained at an incubation of 24 hours (1 day of incubation at 37°C and pH 7.0)(10µg/ml).

Effect of nitrogen source on PHB production

Maximum PHB production was obtained with Yeast as nitrogen source at 37°C and pH 7.0 (8.4 µg/ml). The PHB production was found to decrease with urea, KNO3 and peptone respectively.

Effect of carbon source on PHB production

Maximum PHB production was obtained with mannitol as a carbon source (8.8µg/ml)at 37°C and pH 7.0. PHB production was found to decrease with sucrose, glucose and starch.

Table.1 Morphological Identification of Bacteria

Morphology	PHB 5
Shape	Round
Margin	Curled
Elevation	Flat
Size	Moderate
Texture	Dry
Pigmentation	Absent
Opacity	Translucent

Table.2 Biochemical Characterisation

Sample	Indole Test	M R	V P	Citrate	Starch Hydrolysis	Oxidase	Catalase	Urease	Carbohydrate Fermentation	Protease
PHB 5	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve

Table.3 Optimization of PHB 5 for Maximum Production of Biopolymer

Sample	Parameter		PHB Production (µg/ml)
PHB 5	pH	6	9.90
		7	10
		8	4.80
		9	6.43
	Incubation period	18 hours	4.30
		24 hours	10
		48 hours	8.30
		72 hours	4.90
	Nitrogen Source	Yeast extract	8.40
		Peptone	6.50
		Urea	3.50
		KNO3	2.70
	Carbon Source	Glucose	7.80
		Starch	4.20
		Sucrose	8.70
		Mannitol	8.80

Fig.1 Secondary Screening for PHB Using Sudan Black Staining. The figure shows PHB granules as dark blue, while the cells appeared in pink colour

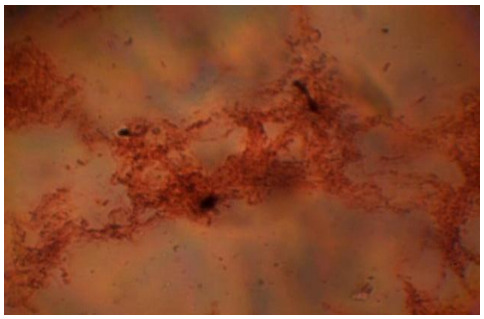
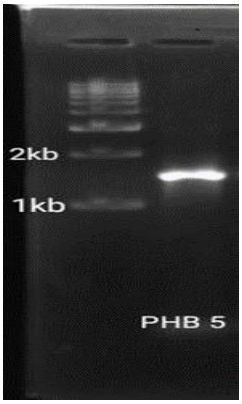


Fig.2 Secondary Screening for PHB Using Nile Blue Staining. The figure shows PHB producing colonies emitting blue coloured fluorescence

Fig.3 Polymerase Chain Reaction. The figure shows an amplicon size of 1500 base pairs (bps)observed in agarose gel electrophoresis after the sequencing with universal primers of 16S rDNA



Lane 1: 1kb marker; Lane 2: DNA isolated from sample PHB 5

Fig.4 Electrophoretogram of PHB 5. The figure shows the composition of base pairs obtained when sequenced with universal primers for 16s r DNA

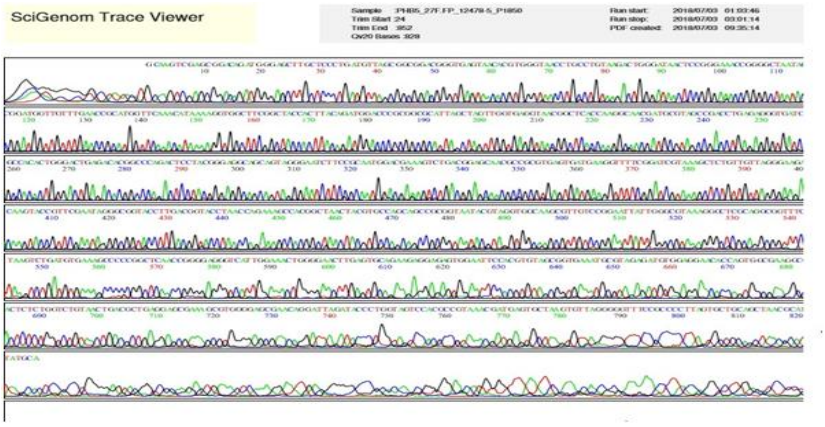


Fig.5 Sequences Showing Significant Alignments of PHB 5 from BLAST search. The figure shows the list of organisms which shows significant alignments to the query sequence

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus subtilis strain P 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MH388434.1
Bacillus subtilis strain JBAR-MP6 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MG835615.1
Bacillus subtilis strain RKMVC 127 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MF939147.1
Uncultured Bacillus sp. clone BYCDW4a 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MG825090.1
Uncultured Bacillus sp. clone BYCDW1a 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MG825089.1
Bacillus subtilis strain LC4 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MH583045.1
Bacillus subtilis R2.6 gene for 16S ribosomal RNA, partial sequence	1480	1480	100%	0.0	100%	LC414178.1
Bacillus subtilis R1.15 gene for 16S ribosomal RNA, partial sequence	1480	1480	100%	0.0	100%	LC414170.1
Bacillus subtilis strain ZB87-1/2 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MH635260.1
Bacillus sp. (in: Bacteria) strain L30 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MH633703.1
Bacillus sp. DM2 chromosome, complete genome	1480	14764	100%	0.0	100%	CP030937.1
Bacillus subtilis strain X4 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MH620013.1
Bacillus subtilis strain SB2 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MH603612.1
Bacillus subtilis strain OTG004 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MH580205.1
Bacillus licheniformis strain FPZSP402 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MG490414.1
Bacillus vallismortis strain FPZSP271 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MG490410.1
Bacillus subtilis strain VITVB1 16S ribosomal RNA gene, partial sequence	1480	1480	100%	0.0	100%	MG755242.1

Fig.6 Phylogenetic Tree of PHB 5. The figure shows the evolutionary relatives of the isolated strain, Bacillus subtilis showed a highest homology with *Bacillus borbori DX 4, Bacillus solani* and *Bacillus licheniformis DSM*

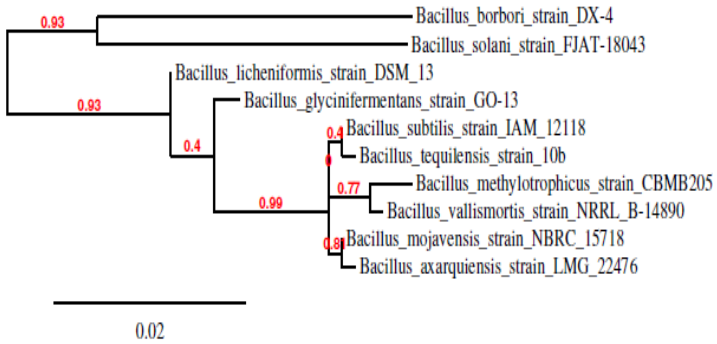


Fig.7 FTIR Spectroscopy. The figure shows FTIR spectroscopy of PHB biopolymer with wave lengths varying from 3471 $^{cm-1}$ to 402^{cm-1}

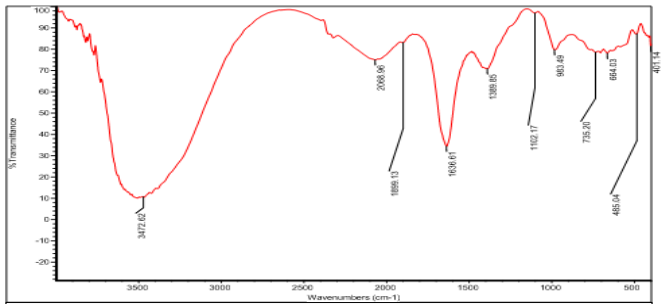
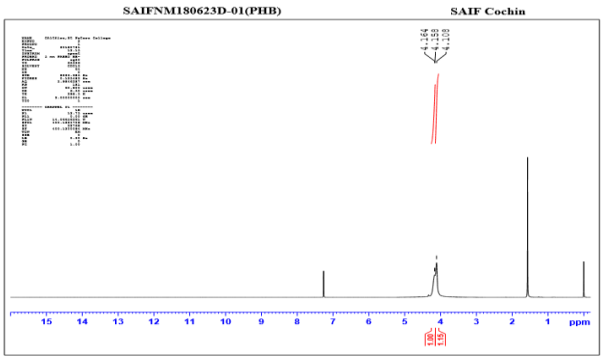


Fig.8 NMR Analysis. The 1H NMR spectral analysis demonstrated the presence of a single proton at chemical shifts 1.5, 4.164 - 4.108 and 7.2 respectively



Molecular Identification of Bacteria

Being highly sensitive and selective, molecular methods are currently used to identify microorganisms. PHB producing strain PHB 5 was further subjected to 16S rRNA primer-based sequencing and analysis. When compared to morphological and biochemical characterization methods, 16S rDNA analysis is found to be the novel and accurate method for identifying unknown species. The DNA from the strain PHB5 was

isolated and the 16S rDNA was amplified using the primers (27F and 1492R) and sequenced. The partial amplification of 16S rRNA was confirmed on the agarose gel electrophoresis. The BLAST analysis of the strain using its 16S rDNA sequence data showed that PHB 5 had highest homology with *Bacillus subtilis*. Using the sequencing data phylogenetic tree was constructed to identify the evolutionary relatives of the isolated strain. The bacterial isolate was identified by analyzing 16S r RNA sequence. By using primers specific for 16S rRNA (27 F and 1492 R), a partial sequence of 16S rRNA gene was amplified. An amplicon size of 1500 base pairs (bps) was observed in agarose gel electrophoresis (Fig3). The size of amplified PCR product was determined by 2.5% agarose gel electrophoresis using DNA markers of 500bp. The isolate was then identified using the maximum aligned sequence through BLAST search. BLAST results indicated 100% identity of PHB 5 sample with *Bacillus subtilis strain P*. The amplified 16S rRNA PCR product was sequenced at SciGenom Labs PvtLtd, Cochin, Kerala using forward and reverse primers (Fig 4). The nucleotide sequence of the amplicon was submitted to NCBI and Phylogenetic tree was generated which shows the evolutionary relatives of the organism. The number at the nodes indicates the percent levels of boot strap based on the analysis of 1000 replicates. *Bacillus subtilis strain p* showed a highest homology with *Bacillus borbori* DX 4, *Bacillus solani* and *Bacillus licheniformis* DSM(Fig5).

FT-IR analysis

The functional groups of the extracted PHA granules were identified by FTIR spectroscopy which is a useful technique to study the functional groups present in the biopolymer. FTIR measurements of PHB biopolymer revealed the bands at 3471-402^{cm-1}. Numerous absorbance bands were observed for the functional groups present in the biopolymer. The specific absorbance bands of the sample corresponding to wavenumbers 3472cm-1, 2088cm-1, 1690 cm-1, 1636 cm-1, 1399 cm-1, 1102 cm-1,983 cm-1,735 cm-1,664 cm-1,495 cm-1, 401 cm-1 were obtained. The functional groups of the polymer already reported were at 1720 (ester C=O valence); 1639 (thioester C=O valence); 1380; 1302; (CH2-S); 1162 (ester C-O).The specific peak at wave numbers 3442 cm−1 attributes to the terminal O-H bonding or water adsorption on the PHB(Fig 7). FTIR measurements of PHB biopolymer revealed the bands at 3471- 402cm-1. The specific absorbance bands of the sample corresponding to wavenumbers 3472 cm-1, 2088cm-1, 1690 cm-1, 1636 cm-1, 1399 cm-1,1102 cm-1,983 cm-1,735 cm-1,664 cm-1,495 cm-1, 401 cm-1 were obtained. The result obtained by this is similar to that of other reports on the biopolymer (De met *et al.*, 1998; Fernandez-Castillo *et al.*, 1986). The functional groups of the polymer reported were at 1720 (ester C=O valence); 1639 (thioester C=O valence); 1380; 1302; (CH2-S); 1162 (ester C-O).The specific peak at wave numbers 3442 cm−1 attributes to the terminal O-H bonding or water adsorption on the PHB (Lopez *et al.*, 2012). The FT-IR spectrum is very closer to the FT-IR spectra of PHB extracted from *Bacillus shackletonii*K5, *B. megaterium* and commercial PHB (Liu *et al.*, 2014; Dhangdhariya *et al.*, 2015; Han *et al.*, 2015).

NMR analysis

The 1H NMR spectral analysis demonstrated the presence of a single proton at chemical shifts 1.5, 4.164 - 4.108 and 7.2 respectively representing $CH_3$and CH groups. The molecular composition of the polymer as indicated by chemical shifts, generates a structure close to (CH_3-CH) backbone (Fig 8).

The resonance of PHB at 1.6 ppm corresponds to the methyl group (CH3). The molecular composition of the polymer as indicated by chemical shifts generates a structure close to (CH_3-CH) backbon.

In conclusion the biopolymer PHB producing bacteria was isolated from rhizosphere soil samples. The bacteria was identified by morphological, biochemical characteristics and further by molecular characterization. The isolate was identified as *Bacillus Subtilis* Strain P by the PCR reaction of r DNA and further by blast search. Synthesis of PHB by the bacteria was confirmed by Sudan black- B staining and Nile blue staining. The characterization of PHB was done by FTIR and NMR analysis. Further the optimum conditions for PHB production were found to be PH of 7.0, an incubation period of 24 hours, Yeast extract as the nitrogen source and mannitol as the carbon source.

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