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Research Article

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Production and Optimization of Polyhydroxybutyrate using *Bacillus subtilis* BP1 Isolated from Sewage Sample

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ABSTRACT

The important biological macromolecule polyhydroxybutyrate (PHB) producing Bacillus subtilis *was isolated from the sewage sample and identified by means of 16S rRNA analysis. The renewable nature and biodegradability of PHBs make them suitable material to replace synthetic plastics in many applications. Presently their production is exclusive, but these plastics are only in the initial stage of commercial development. In the present study the physico-chemical parameters such as Time, Temperature and pH were optimized for increased production of PHB. It was found that maximum production of PHB was observed at 48 hours of incubation time (328µg/ml), temperature of 37˚C (336µg/ml) and pH of 7.5 (362 µg/ml). The low cost production media was developed using fruit peel extract and sugarcane industrial waste. The amount of PHB produced using low cost production media with fruit peel was 234 µg/ml and with sugar industrial waste was 218 µg/ml. The PHB produced was characterized using FTIR and NMR analysis.*

Keywords: *Polyhydroxybutyrate, Bacillus subtilis, optimization.*

INTRODUCTION

Humans have led to the increasing number of non-degradable waste materials across our planet. The environmental pollution caused by indiscriminate dumping of plastic waste has unsaid global proportions. The uses of plastic are found in all industries, especially for packaging and they account for more than 50 % of total consumption for bottling and baling. They cause serious problems to the environment, i.e., the accumulation of non degradable plastics, incineration contributing to greenhouse gas emission. Incineration of plastic generates toxic molecules such as dioxin that are more harmful than the plastic. Recycling is a laborious process and materials can only be recycled for a limited number of cycles before they lose their mechanical properties. Synthetic polymers are xenobiotic and are recalcitrant to microbial degradation.. Increased environmental problems associated with discarding of plastic lead many studies towards the development of a suitable eco-friendly material that can replace at least some of the commodity plastics. Biodegradable plastics are an alternative to avoid the pollution problems caused by commercial plastics[1].Bioplastics are natural biopolymers that are synthesized and catabolized by various organisms[2] and these materials do not cause toxic effects in the host and have certain advantages over petroleum-derived plastic[3]. These biopolymers accumulate as storage materials in microbial cells under stress conditions[4]. Bio-plastics have desired physical and chemical properties as that of conventional synthetic plastics. Bio-plastics developed from renewable agricultural or biomass feedstock mimics the properties of conventional plastic and are degradable through microbial processes upon disposal. Biological plastics includes starch-based plastics, protein based plastics, and cellulose-blended plastics. Biological plastics can be blended with conventional plastics such as polyethylene (PE), polypropylene (PP), and poly (vinyl alcohol). However, such bio-based plastics are only partially biodegradable.

Polyhydroxybutyrate (PHB) is the most studied member of the polyhydroxyalkanoate (PHA) family. PHB is synthesized by cells under limiting growth conditions, when the carbon source is in excess and nitrogen, phosphorus, magnesium, oxygen or sulfur are in limited concentration. PHB is attracting considerable attention because of their potential as renewable and biodegradable plastics in the areas of tissue engineering, environmental friendly packaging materials, and as a chiral hydroxyalkanoate pool.

MATERIALS AND METHODS

Sample Collection and Isolation of PHB Producing Bacteria

Sewage samples were collected from Adyar, Chennai which were taken to laboratory for experimental work. 1.0 ml of sewage sample was serially diluted from 10^1 to 10^7 dilutions and the diluted samples were plated on Nutrient agar plates by spread plate method. The plates were incubated at 37°C for 24 hours. The isolated strains were maintained on nutrient agar slants and stored at 4°C.

Screening of the polyhydroxybutyrate granules

The Isolated strains were screened for the presence of Polyhydroxybutyrate granules using Sudan B black staining technique[5]. The positive cultures showing dark purple colour polyhydroxybutyrate granules were maintained separately in nutrient agar slants and among them one was selected for further PHB production studies.

Identification of the Isolate: The identification of the isolate was performed using Bergey's manual.

Productionof Polyhydroxybutyrate (PHB)

Preparation of Inoculum: The primary inoculum was prepared in Luria Bertani medium in 250 ml conical flask containing 50 ml of sterile medium and inoculated from the stock culture. The fresh over night culture was used as an inoculum for production of polyhydroxybutyrate.

Determination of PHB

The spectrophotometric chemical assay for the determination of PHB from the sample was estimated using Law and Splepecky method 1961.

Digestion method: After centrifugation at 10000 rpm for 20 mins the pellet was lyophilized. Then the lyophilized pellet was digested with 30% sodium hypochlorite solution at 37°C for 20 min. The collected residue was centrifuged at 8000 rpm for 20 min and a series of washing was carried out using distilled water, acetone and ethanol. The polymer was dissolved in chloroform and kept for complete evaporation. 5 ml of concentrated H_2SO_4 was added and heated for 40 min at 100° C in a water bath. The resultant crotonic acid was determined at 235nm against H_2SO_4 as blank using UV –VIS spectrophotometer. The concentration of PHB was calculated by comparing the OD value with a standard graph prepared using P3HB (Sigma Aldrich)[6]. The PHB positives isolates were studied for the production ability in the standard production medium. Among the isolated strains, the strain which gave the maximum production was chosen for further optimization studies.

Gene sequencing: The gene sequencing was carried out using Beckman Coulter CEQ 8000 auto analyzer. (The original sequencing was done at Delhi university south campus). QIAQuick (Qiagen) Spin column was used to clean the amplified products and DTCS quick start Dye terminator kit (Beckman Coulter) was used to carry out the cycle sequencing.

16S rRNA sequence analysis: Sequence alignments provide a powerful way to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be inferred from well designed queries and alignments. BLAST – Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/) provides a method for rapid searching of nucleotide and protein databases. As BLAST algorithm detects local as well as global alignment, regions of similarity may provide information related to the function of uncharacterized nucleotides and proteins.

Optimization of physico-chemical parameters for production of PHB: The influence of various time course, pH levels and different carbon & nitrogen sources on the overall PHB production were investigated using Production medium[7].

Effect of incubation time on PHB production: 500 ml of sterile production medium was prepared and 1% inoculum was added aseptically. The inoculated medium was incubated at 37°C temperature with shaking at 100 rpm. 10 ml of culture was aseptically drained periodically at 6 hours intervals up to 72 hours. The PHB production was determined using alkaline digestion method (Nisha *et al.,* 2009).

Effect of Temperature on PHB production: 100 ml of sterile production medium was prepared in different conical flask and inoculated with 5 % inoculums. Each flask were incubated for 48 hours at different temperatures such as 27ºC, 32ºC, 37ºC, 42ºC and 45ºC and PHB concentration was determined.

Effect of pH on PHB production: 100 ml of sterile production medium was prepared in different conical flasks and each flask was adjusted to different pH such as 6, 6.5, 7, 7.5, 8 using 0.1N NaOH and 0.1N HCl. The flasks were inoculated with 5 % inoculum and incubated for 48 hours at 37ºC after sterilization. The PHB production was estimated.

Development of low cost Production Media: Fruit peel waste of sapota and waste water from sugar industry were collected for research work. The fruit peel waste was shade dried for 5 to 7 days and was powdered with a pre-cleaned grinder, then sieved and used for preparation of extract. The sugar component of the production media was replaced by fruit waste extract and used for PHB production. Similar production medium was prepared amended with waste water from sugar industry for PHB production.

Effect of fruit peel waste and sugar industry waste on PHB production: The fruit extract and sugar industry waste water were amended with the production medium separately at different concentration such as 10% to 100% . After sterilization, the flasks were inoculated with 1% inoculum and incubated at 37ºC for 48 hours. The PHB production was determined using alkaline digestion method (Nisha *et al.,* 2009).

Fourier Transform-Infrared Spectroscopy: FTIR is one of the rapid and powerful tools to obtain information on polymer structure, because every chemical compound in the sample makes its own distinct contribution to the absorbance/transmittance spectrum. After 48 h the cells were harvested and PHB was extracted using chloroform and cold methanol dried and subjected to FT-IR spectroscopy. The samples were prepared in KBr pellet and FT-IR (Bruker) absorption spectrum was measured in room temperature of range 4000–600cm $^{-1}$. The absorbance peak values obtained were compared with the available literature values and the sample was confirmed for the presence of PHB.

Nuclear Magnetic Resonance (NMR): The polymer was suspended in spectro chem grade deuteron chloroform ($CDCl_3$). The ^{1H} NMR spectra of sample was obtained at the range of 0-13 MHz while that of 13C NMR spectra in the range of 0-200 MHz using a Bruker AV III NMR spectrometer (Bruker BioSpin AG, Switzerland) from Sophisticated Analytical Instrument Facility, IIT, Madras. The spectra thus obtained were compared with the standard NMR spectra to confirm the presence of C atoms.

RESULTS

Sample Collection and Isolation of PHB producing bacteria

In this study, Bacterial strains were isolated from sewage soil samples collected at Adyar, Chennai. The samples were serially diluted and plated on Nutrient agar medium. Sixteen bacterial isolates were obtained.

Screening for PHB Granules

The 16 isolates selected were screened for the presence of polyhydroxybutyrate using sudan black staining method. Among them 5 gave positive results for PHB production (Figure.1).

PHB production: The screened isolates were cultivated i n the production medium and the amount of PHB produced was estimated. Among the 5 isolates, strain designated as BP1 produced maximum amount of PHB of 318 µg/ml. This strain was taken for further identification and optimization studies.

Identification: The selected isolate BP1 was identified based on t he Bergey's classification and it was found to be *Bacillus subtilis* (Figure. 2).

Molecular identification of *Bacillus subtilis:* The potential PHB producing bacteria, *Bacillus subtilis* was ascertained its systematic position based on 16S rRNA sequence analysis, BLAST homology analysis was also carried out to compare with other 16S rRNA sequences available in the GenBank of NCBI. It revealed the sequence of *Bacillus subtilis.*

Figure 1 : Microscopic apperance Sudan Black B staining of *Bacillus subtilis*

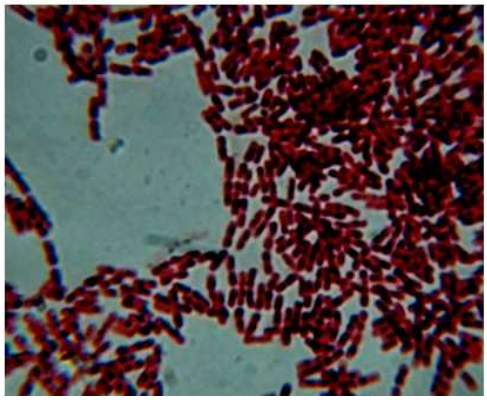


Figure 2 :Plate shows *Bacillus subtilis* colonies in Nutrient Agar Medium

16S rRNA sequence analysis: The 16s rRNA was amplified and sequenced the partial sequence of 16S r RNA was obtained (Figure. 3).

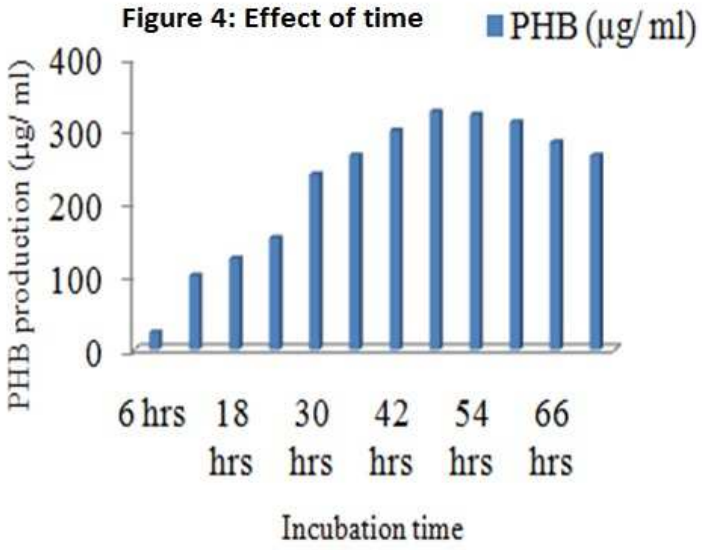
Figure 3: 16S ribosomal RNA gene, partial sequence (1267 bps) of *Bacillus subtilis* BP1

TTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGACAG
ATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGTCCGGGAAACC
GGGGCTAATACCGGATGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGCCTAC
CACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGTGGAGGTAACGGCTCACCAAGGCAA
CGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACT
CCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTT
CGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGG
CGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGG
GAACTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT
GGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT
GTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTA
CGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAG
ATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTGCCAGCATTCAGTT
GGGCACTTTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCAT
CATGCCCCTTATGACCTGGGCTACACACCTGCTACAATGGACAGAACAAAGGGCAGCGAAA
CCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCA

Optimization of PHB production

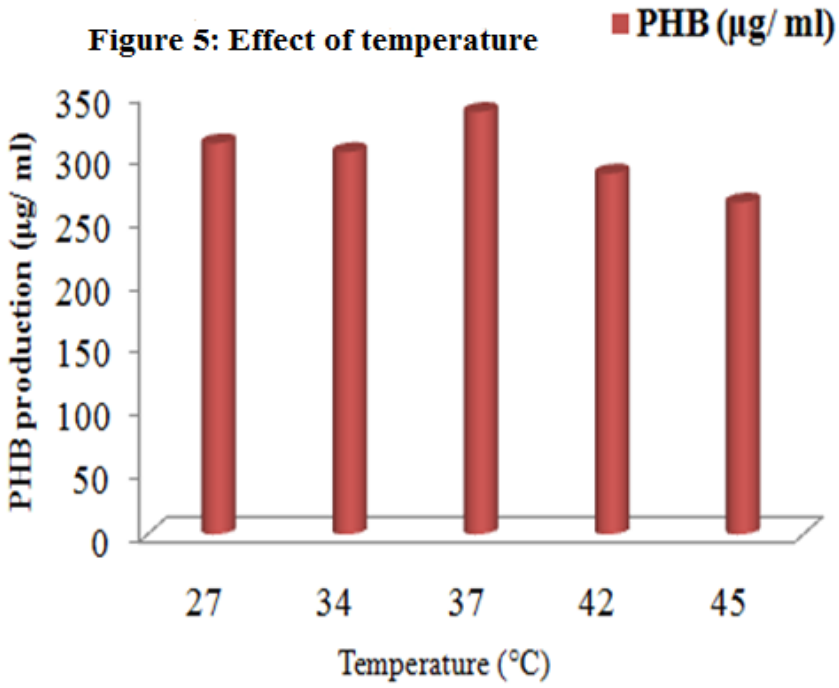
Effect of Incubation Time:

The selected isolate was cultivated in LB medium. The PHB production at various incubation time were analyzed and tabulated. A maximum of 328 µg/ml PHB production was observed at 48 hour incubation (Figure 4).



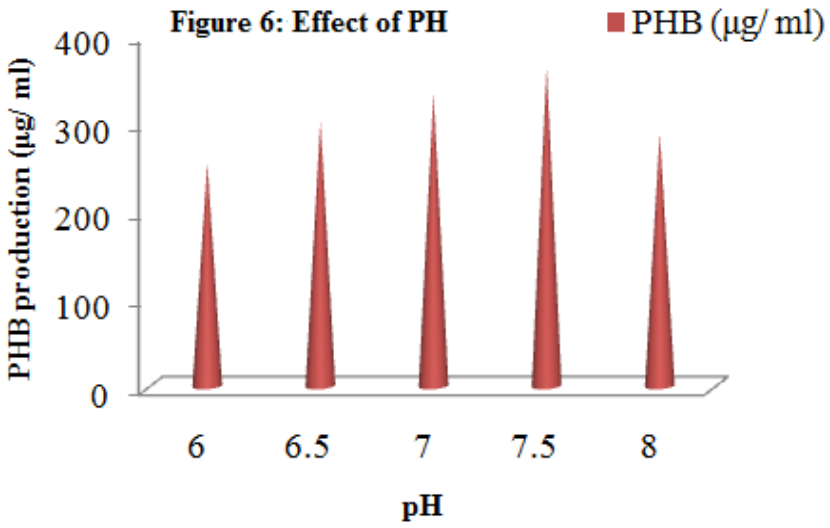
Effect of Temperature

The isolate was cultivated in LB medium and incubated at various temperatures. From Figure.5 it is observed that maximum amount of PHB (336 µg/ml) was produced at 37^0C incubation.



Effect of pH

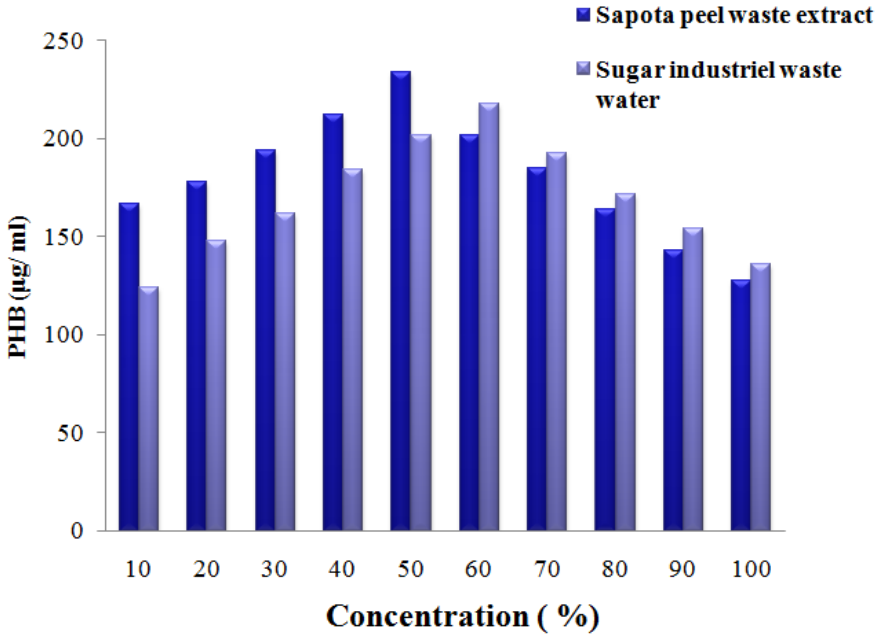
The isolate was cultivated in LB medium at various pH and incubated. From Figure.6 it is observed that maximum amount of PHB (362 µg/ml) was produced at pH 7.5.



Development of low cost Production Medium

In this study, Sapota fruit peel extract and waste water from sugar industry have been used as substrates for PHB production. The results revealed that, maximum production was observed in fruit peel extract when compared to waste water from sugar industry (Figure.7). Extracts were subjected to further optimization in which different concentration of fruit peel extract and sugar industry waste water were mixed separately with standard production medium for the PHB production. From the figure 7 it is evident that there is gradual increase in the PHB production with increasing concentration of Sapota peel extract and sugar industry waste water in the medium. The result shows maximum production was obtained at 50% of fruit peel extract and at 60% of sugar industry waste water respectively. The amount of PHB produced using low cost production media with fruit peel was 234 µg/ml and with sugar industrial waste was 218 µg/ml.

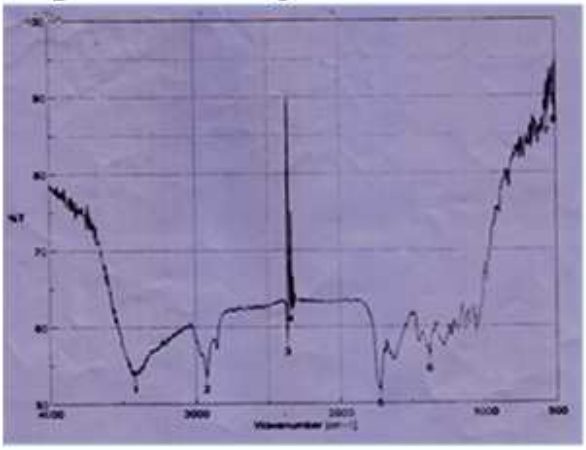
Figure 7: Effect of Agro products on PHB Production



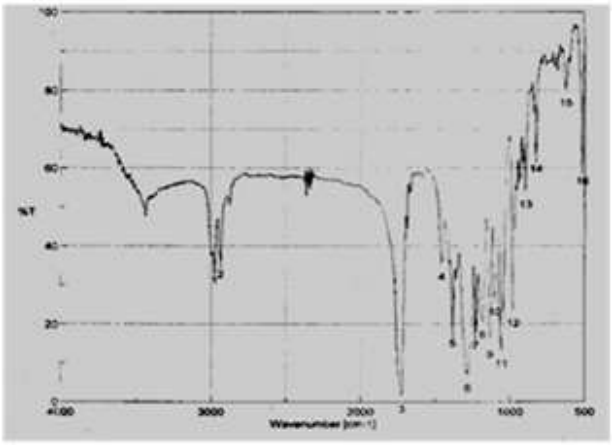
Fourier Transform - Infrared spectroscopy

Figure 8. shows the FTIR spectrum of partially purified PHB. A strong adsorption band was observed at 1726.94 cm^{-1} and other adsorption bands were formed at 2343.09, 1399.1, and 507.187. The absorbance peak values obtained were compared with earlier reports to confirm the polymer compound. The FTIR spectrum of partially purified PHB obtained from Starch water is shown in Figure 8 (a & b). There was a strong adsorption band at 512.008 cm^{-1} and other adsorption band peaks were observed at 2345.98, 2366.23 and 561.184.

Figure 8: Infrared spectrum obtained



a) PHB produced by sapota peel extract



b) PHB produced by sugar industries

NMR analysis

NMR analysis was used to determine quality of structural composition of PHB. The 1H and 13C NMR spectra obtained from PHB samples produced using sapota peel extract and sugar industry waste water were shown in Figure 9.These spectra were compared with the commercial PHB (Fluka, Sigma-Aldrich Chemicals, USA). Both the Spectra matched with each other and the peaks observed in the spectra overlap representing different types of carbon atoms present in the PHB structure, [-O - CH - (CH_3) - CH_2 - (C =O) -] *n.* The chemical shift signals of 13C NMR spectrum obtained in the present work were agreed with the the commercial PHB[8].

Figure 9: ^{1}H NMR spectra of PHB produced from *Bacillus subtilis*

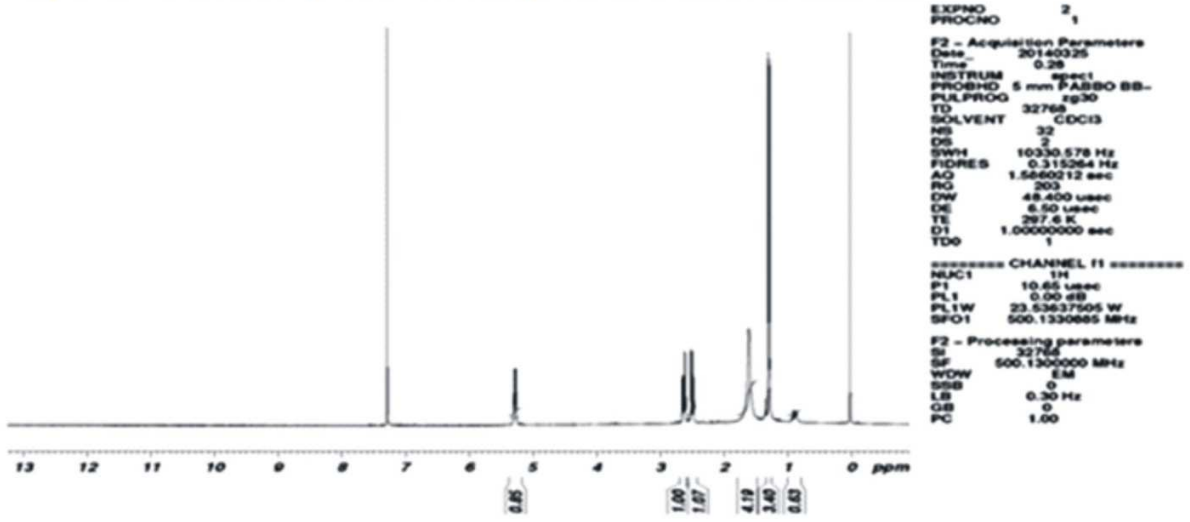
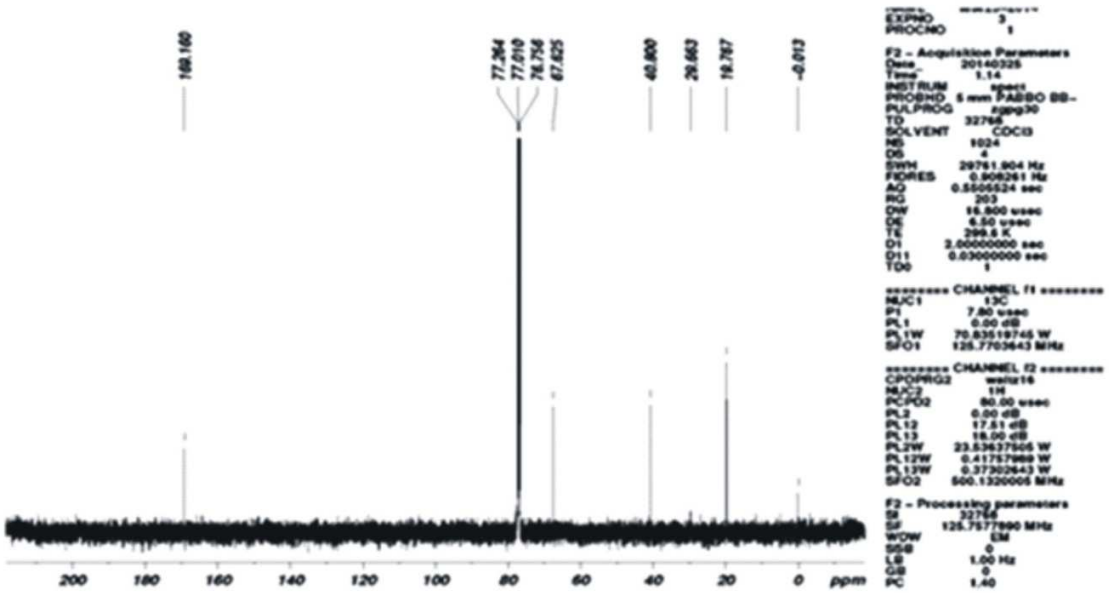


Figure 10, 11 : ^{13}C NMR spectra of PHB produced from *Bacillus subtilis*



All the carbon atom peaks obtained by PHB produced from *Bacillus subtilis* were in accordance with the standard peak. The CH_3 atom peak was formed at 19.76 in sample while it was formed at 19.65 in the standard. Similarly the CH_2, CH and C=O atom's peak of the sample was obtained at 40.80, 67.62, and 169.16 while that of the standard peak was obtained at 40.66, 67.48, 169.03 respectively. (Figure 10,11)

The figure showed a number of characteristic PHB peaks at δ = 4.19, 3.40, and 1.07 corresponds to a ─ CH doublet, ─ CH_2 multiplet, and –CH_3 respectively. Two additional small peaks were formed at δ = 0.63 and δ = 1.00 due to impurities present[9]

DISCUSSION

Contradictory effects of incubation time on PHB production by various microorganisms have been reported. Nur *et al.,* 2004 in their investigation found that a best PHB production was observed after 45 hours. Sangkharak, and Prasertsan, 2008, indicated that PHB was a growth associated product and its accumulation significantly increased when all cultures reached the exponential phase (after 18 hrs) till stationary phase[10]. To assess the effect of time on the production of PHB and reported that *Brevibacterium casei* SRKP2 shows the maximum PHB production (0.135 g/L) at 48 hours. Ram Kumar et al., 2009, reported the optimum growth was observed at pH 8.0. Variation in initial pH of the medium showed a strong influence on the production of PHB. Even a slight variation in pH from the optimum point denoted a sudden reduction in PHB production. Initial pH value of 7.5, gave the maximum production of PHB of 25%. Although PHB production was enormous in earlier studies, the raw materials used are cost effective. For the production of PHB, the cost of the carbon source is supposed to be cheap and yield should be the maximum[11].

A strong peak at 2345 cm^{-1} indicates the stretching of C O ester bond of Polyhydroxybutyrate, . Also the other peaks obtained at 1381 cm^{-1} (corresponding to C H stretch), 3422 cm^{-1} (corresponding O H stretch) and 2345 cm^{-1} (corresponding to C =C stretch), are much closer to the identified peaks for PHB .Analogous results were reported by Desouky *et al.*, 2007 and Macid Nurbas showing strong adsorption band at 1279 cm^{-1} and other adsorption bands at 1379, 1454, 2928, 1724 and 3750 cm-1 for - CH3, -CH2, -CH, C=O, and O-H groups respectively[12,13].

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