

Original Article

CHARACTERIZATION OF POLYHYDROXYBUTYRATE SYNTHESIZED BY *BACILLUS CEREUS*

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ABSTRACT

Objectives: To characterize the Polyhydroxybutyrate (PHB) produced from *Bacillus cereus* using various instrumental methods and reduce them to the nanoscale which can be used as drug carrier

Methods: The isolated bacterium was identified by 16S rDNA analysis. PHB produced by the bacterium was extracted by a process using boiling chloroform followed by methanol which selectively precipitates PHA. The polymer produced was analyzed using various instrumental techniques like FTIR, GC-MS, ¹H and ¹³C NMR, XRD and FESEM. This biogenic PHB has been reduced to nanospheres which were analyzed by FESEM.

Results: A bacterium with the ability to produce PHB was isolated from the cloth used to smear oil on pan cake pan which was identified as *Bacillus cereus* by 16S rDNA analysis. The organism was capable of accumulating 1.19 g L⁻¹ of PHA corresponding to 49.7% of its dry weight after 48 h of incubation. The polymer produced was analyzed using various instrumental techniques which identified the polymer as PHB.

Conclusion: The organism seems to be a potential candidate for the biogenic synthesis of PHB which can find application as drug carrier.

Keywords: *Bacillus cereus*, Growth, Biodegradable PHB, Characterization, Nanoparticles.

INTRODUCTION

Plastics have become a part of human life. Plastics have become indispensable as it can be found everywhere and everything. Many industries will become non-existent in the absence of plastics. Their uses in the medical field further reiterate their importance. They are key components of modern prosthetic devices, surgical gloves, contact lenses, disposables which prevent infections [1]. Further they serve as a wound dressing material, surgical pins, sutures and swabs, and as carrier for long term dosage of drugs [2]. Many of today's most innovative medical procedures are dependent on the use of plastics.

More than 50% of plastics consumed in India land up as landfills posing deep threat to the environment as they are non biodegradable and highly inert nature. Vast application and usage of plastics in packaging is also a barrier to put a full stop on the usage of same. Plastics are now depicted as an ineluctable burden on mankind as these are difficult to recycle and properties of these plastics deteriorate after recycling [3].

Bioplastics or Green plastics are plastics which have their origin from biological sources such as vegetable fats and oils, corn starch, pea starch or microorganisms. Polyhydroxyalkanoates (PHA), a family of biopolyesters with diverse structures, are the only bioplastics completely synthesized by microorganisms [4]. Polyhydroxyalkanoates (PHAs) are natural, thermoplastic, aliphatic biopolyesters that fully comply with requirements like *biobased*, *biodegradable*, *compostable* or *biocompatible* to qualify them as *Green plastics*. Among all bio-based plastics, they are unique by being entirely produced and degraded by living cells naturally and completely to CO₂ and H₂O under natural environment [5].

Several heterotrophic and autotrophic aerobic bacteria synthesize and accumulate PHA as carbon and energy storage materials under the condition of limiting nutrients in the presence of excess carbon source [6-10]. *Bacillus* species were studied by number of researchers for their ability to synthesize PHA. In one of the study, 29 *Bacillus* species have been isolated and screened for their ability to produce PHB in which Yilmaz et al. [11] have reported that *Bacillus brevis* followed by *Bacillus cereus* accumulated appreciable amount of PHB qualifying them as a potential candidate for

industrial application. Wu et al. [12] have reported that *Bacillus* sp. JMa5 strain accumulated 25-35%, (w/w) PHB during sucrose fermentation. *Bacillus cereus* studied in this work has the ability to produce appreciable amount of PHA. The PHB production by *Bacillus cereus* increased from 34.9% to 52.4% (1.2 g of PHB l⁻¹) (w/w) after optimization [13].

MATERIALS AND METHODS

Materials

The chemicals used for preparation of reagent, solutions and microbiological growth media were purchased from Hi-Media Laboratories Pvt. Ltd. Mumbai, India and SISCO Research laboratory, Mumbai, India. Solvents used in the studies were of AR grade and were purchased from Merck Pvt. Ltd.

Microorganisms

Bacillus cereus showing appreciable PHA production was isolated from oil amended clothes (used to smear oil on pan cake pan). The bacterium was identified based on their biochemical and molecular characterization. The PCR amplification and DNA sequencing of the 16S rDNA gene fragment of the bacterial strains were carried out by isolating genomic DNA from the pure culture pellet. The ~1.4kb rDNA fragment was amplified using high - fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the forward, reverse and internal primer. The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors [14, 15].

Culture conditions

The mineral salt medium used in the initial screening studies for PHA production was of the following composition (g/L) 2.5 K₂HPO₄, 2.5 KH₂PO₄, 5 glucose, 2 yeast extract, 0.2 MgSO₄·7H₂O, 0.01 FeSO₄·7 H₂O and 0.007 MnSO₄·7 H₂O. The pH of the medium was adjusted to 7.5. The medium without glucose was sterilized at 121°C for 20 min. Glucose was sterilized separately and added to the medium.

PHA extraction and quantification

The bacterial cells after 48 h of incubation were harvested by centrifugation at 5, 000 rpm for 10 min and were dried. The dry cell weight was taken as an index of biomass (CDW). The dried cells

were treated with boiling chloroform to dissolve the PHA along with lipids. This solution was filtered using Whatman No.1 filter paper to removed the cell mass. The filtrate was treated with methanol which selectively precipitates PHA from the solution [16]. This solution was further subjected to centrifugation at 12,000 rpm for 10 min to precipitate the PHA and was air dried. The dried PHA was weighed and stored for further studies.

Instrumental analyses

FTIR: The sample in chloroform was applied as a smear over the NaCl block and was analyzed using Perkin Elmer RX1 FTIR spectrophotometer. The FTIR spectrum of the sample was obtained at the wavelength in the range of 450-4000 cm⁻¹.

GC-MS: GC-MS analysis of the sample was carried out after methanolysis of PHA [17]. For methanolysis of PHA, polymer sample was suspended in 1 ml chloroform and 1 ml methanol containing 2.8 M H₂SO₄ in a screw capped tube, and then incubated at 100°C for 2 h. After cooling, 0.5 ml demineralized water was added, and then the organic phase containing the resulting methyl esters of 4-hydroxyalkanoic acids were analyzed by using GC-MS-QP 2010 [SHIMADZU] MS spectrometer. The column used was VF-5ms, 30 m x 0.250 mm dia with the film thickness of 0.25 µm and the column oven was programmed between 70 and 300°C at the rate of 10°C per minute with the injection temperature of 240°C. Mass spectra were recorded under scan mode in the range of 40-1000 m/z. Compounds were identified using NIST11. L Library.

NMR: Nuclear Magnetic Resonance spectrum ([13]C) of biogenic PHB (40 mg/ml) was recorded at 100 MHz using CDCl₃ as solvent and the ¹H-NMR spectrum was recorded at 400 MHz at 24°C using Bruker Spectrometer.

SEM: SEM analysis was carried out to understand the size and morphology of *Bacillus cereus*, the PHB synthesized and the microspheres using Field emission Scanning electron microscope (Carl Zeiss, Germany).

XRD: To get an insight into the structure of PHA produced by the organism, powder XRD patterns were recorded using an X-ray

diffractometer (Rigaku, Japan) using Cu K-beta (30 kV, 100 mA) radiation. Data were recorded in 2θ range of 10°-80° under continuous scan mode using the scan rate of 4°/min

Synthesis of PHB Nanoparticles

Large PHB nanoparticles were synthesized using this biogenic PHB using the method described by Xiong et al. [18]. Briefly, 50 mg of hydrophobic polymers was added into 1 ml dichloromethane, and the mixture was stirred to ensure that all materials were dissolved. 20 ml of 1% PVA (w/v) was sonicated for 1 min and was slowly added with 1 ml of organic solution. Ultrasonication (Vibra cell V 501, Sonics, USA) was continued for 10 min. The mixture was gently stirred for 3-5 h at room temperature. The nanoparticles were collected by centrifugation at 1.5 × 10⁴ × g for 10 min, followed by washing twice with deionized water.

RESULTS AND DISCUSSION

Identification of Microorganism

The bacterium showing appreciable amount of PHA production was isolated from an oil dipped cloth used to smear oil on pan cake pan. The bacterial isolate was initially characterized using various microbiological and biochemical tests and was identified to be gram positive *Bacillus* sp.

This was further confirmed by 16S rDNA analysis. Since the 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification it has become the most reliable technique for bacterial identification.

BLAST program was used to compare the 16s rDNA sequence of the isolate with the database sequences to obtain the sequences that displayed maximum similarity with that of the query. All the sequences reported by BLAST revealed that the isolate showed high percentage of similarity (95%) with the sequence of *Bacillus cereus* with a reasonably high score and e-value being zero. The 16s rDNA sequence and the phylogenetic tree that was constructed for these possible homologs using neighbor joining method are showed in fig. 1.

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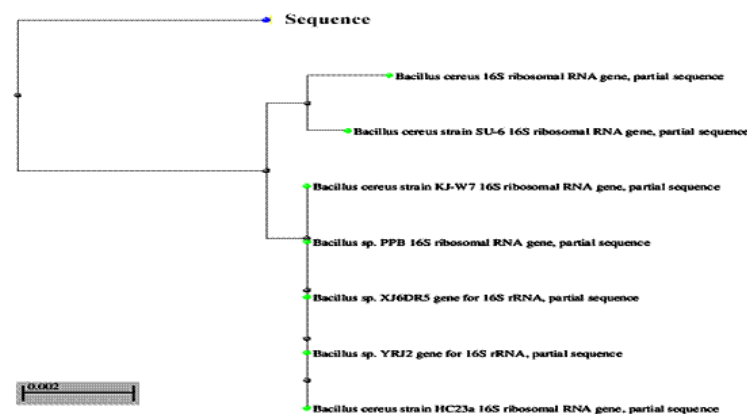


Fig. 1: The 16s rDNA sequence and Phylogenetic tree depicting the relation between the 16s rDNA sequence of the isolate with its possible homologous sequences

Growth and PHA production

The bacterial growth and PHA production by *Bacillus cereus* was followed simultaneously and the results are given in fig. 2. The bacterial growth and PHA production by *Bacillus cereus* were followed simultaneously and the results are given in fig. 2. Growth as well as PHA production increased up to 48 h of incubation reaching a maximum of 3.4 g l⁻¹ and 1.19 g l⁻¹ respectively after which they started to decrease.

Bacillus cereus exhibited a growth rate of 88 mg h⁻¹ by 24 h of incubation which decreased to 70 mg h⁻¹ after 48 h⁻¹. The rate decreased slowly upto 34 mg h⁻¹ by 96 h of incubation and by 144 h of incubation it reached 22 mg h⁻¹. With reference to PHA production rate of production was 24 mg h⁻¹ by 24 h of incubation which decreased by 41% after 48 h and by 75% after 120 h of incubation. The decrease in the concentration of PHA may be due to the degradation of polymer intracellularly by endogenous PHA depolymerase [19, 20].

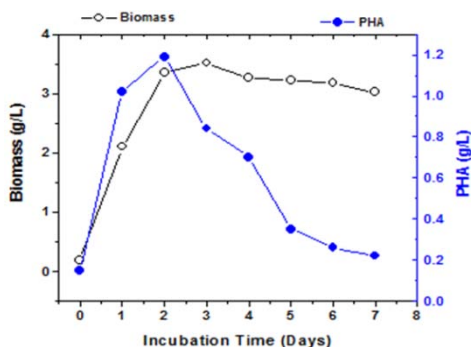


Fig. 2: Growth and PHA production by *Bacillus cereus*

Characterization of PHA

The FTIR spectrum of the PHA (fig. 3) has shown prominent peaks at different wavelengths which are characteristics of Polyhydroxybutyrate. The strong and broad peak at 3436 cm⁻¹ clearly indicates the presence of O-H stretching of alcohol whereas the strong peaks at 2975 and 2932 cm⁻¹ are due to the C-H stretch of alkanes. The presence of C=O and C-O stretch of ester could be confirmed from the peaks at 1725 cm⁻¹ and from the series of intense peaks located at 1101 cm⁻¹ respectively [21-24]. The FTIR spectrum of the biogenic PHA compares well with the FTIR spectrum obtained by Oliveira et al. [25] for the standard and sample PHB.

The GC-MS spectra of methanolized PHA (fig. 4) showed 5 prominent peaks with RT values of 3.33, 4.625, 10.49, 11.83 and

12.02 min corresponding to different derivatized products of Butenoic acid confirming the presence of polyhydroxybutyrate. The m/z signals corresponding to each R_T values and the compound identified are given in table 1. Kim et al. [26] have confirmed the presence of 3HB monomer produced by Poly(3-hydroxybutyrate) depolymerase from a Fungal Isolate, *Emericallopsis minima* W2 using GC chromatogram that contained a peak corresponding to the R_T value of 4.99 min, with ion fragments having m/z values of 43, 60, 71, and 89.

In another study, Nurbas and Kutsal [27] have confirmed the presence of PHB using GC-MS spectra where a characteristic peak with the R_T value of 3.61 min with ion fragments having m/z values of 43, 59, 74, 87 and 102.

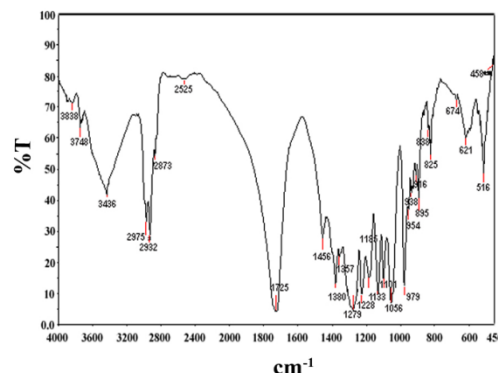


Fig. 3: FTIR spectrum of PHB produced by *Bacillus cereus*

Table 1: The m/z signals corresponding to each R_T values and the compounds identified

R _T	m/z	Compound identified
3.3	117, 103, 86, 68, 57, 41	2 Butenoic acid
4.63	96, 86, 68, 57, 41	2 Butenoic acid
10.49	117, 100, 85, 69, 59, 41	2 Butenoic acid, Methyl ester
11.83	128, 103, 87, 69, 55, 41	2 Butenoic acid, Methyl ethyl ester
12.02	136, 126, 113, 103, 87, 69, 58, 41	2 Butenoic acid, Ethyl ester

¹³C NMR spectrum of the biogenic PHB (fig. 5a) has shown four important peaks indicating the presence of different types of carbon atoms (C=O, CH, CH₂ and CH₃) in the PHB. The chemical shift signals obtained are almost similar with the chemical shift signals obtained for PHB produced *Bacillus megaterium* and *Cupriavidus necator* as reported by Doi et al. [28] and Oliviera et al. [25] respectively. The

structure of biogenic PHB was further confirmed from the ¹H NMR spectrum (fig. 5b) which showed prominent peak at 1.2 ppm that may be attributed to the presence of CH₃ side chain. A series of peaks at 2.4-2.6 ppm clearly indicate the presence of CH₂ whereas the peak at 5.2 is due to the presence of CH group.

This spectrum correlates well with the ¹H NMR spectrum reported by Abd-El-Haleem et al. [29] for the PHB synthesized by two transgenic yeasts *S. pombe* Q01 and *S. cerevisiae* INVSc1. Wu et al. [30] have observed the signals attributed to PHB part at 1.27, 2.35–2.71, 1.5–1.70, 5.21–5.30 and 0.9. The chemical groups and their chemical shift signals for ¹³C NMR and ¹H NMR spectra are given in table 2.

Table 2: Chemical shift signals obtained in ¹³C NMR and NMR spectra obtained for PHB produced by *Bacillus cereus*

Group	¹³ C	¹ H
CH ₃	19.77	1.2
CH ₂	40.80	2.4-2.6
CH	67.63	5.2
C=O	169.16	-

The XRD study was carried out to check crystalline structure of PHB. The XRD diffractogram (fig. 6a) showed four prominent peaks at 13.69°, 17.32°, 22.67°, 25.32 and 44.5°. The presence of intense peak at 13.69° indicates the crystalline nature of the polymer. The diffractogram is almost identical with that obtained by Oliveira et al. [25] for the standard PHB as well as for the PHB produced by *Cupriavidus necator* by soil-state fermentation. Similar results for the diffractogram of PHB were reported previously by few researchers [31, 32]. The crystalline nature is further confirmed from the FESEM micrograph (fig. 6b) of PHB produced by *Bacillus cereus*.

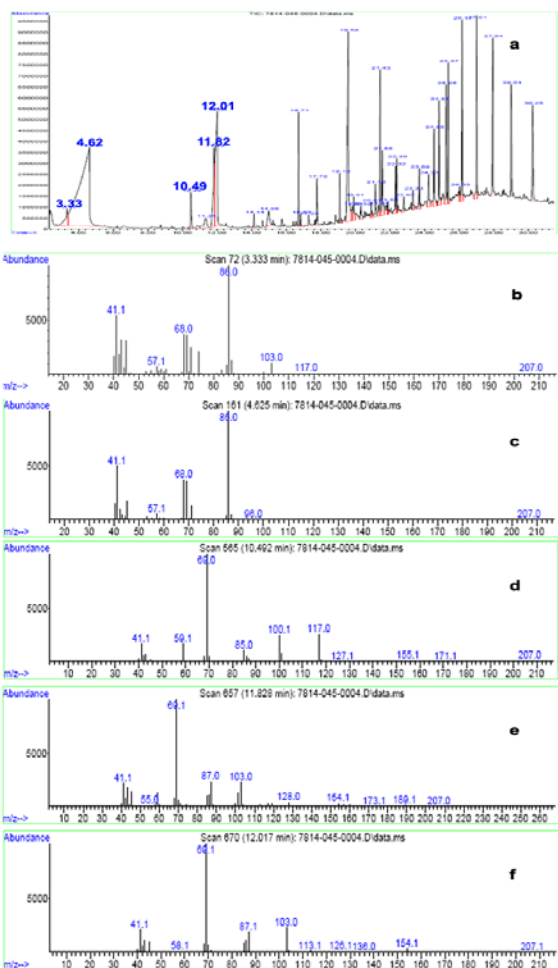


Fig. 4: GC-Mass Spectra of PHB produced by *Bacillus cereus* (a) Gas chromatogram and mass spectra for the peak corresponding to R_T value of (b) 3.33 min (c) 4.63 min (d) 10.49 min (e) 11.83 min and (f) 12.02 min

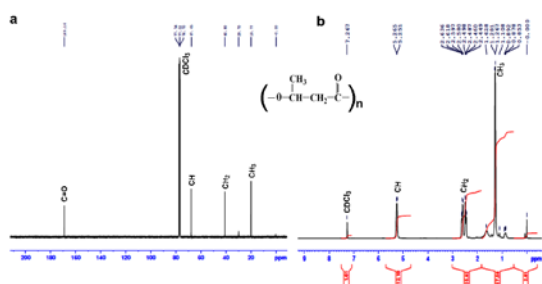


Fig. 5: (a) ¹³C NMR and (b) ¹H NMR spectra of PHB produced by *Bacillus cereus*

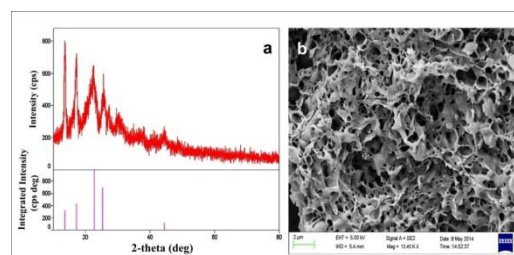


Fig. 6: (a) X-ray diffractogram and (b) Scanning Electron Microgram obtained for PHB produced by *Bacillus cereus*

PHB nanoparticles

PHB nanoparticles with the size ranging 200–350 nm (fig. 7) were prepared by ultrasonication method. Nanoparticles prepared by these methods showed smooth surfaces free of major defects similar to the large nanoparticles synthesized by Xiong et al. [18]. But in contrast to his report the size was quite larger than the nanoparticles described by him which was in the range of 150–300 nm. These nanoparticles could be seen as spherical structures embedded in the PVA matrix. This procedure further requires modifications which can yield smaller nanoparticles with uniform size and with lesser PVA matrix.

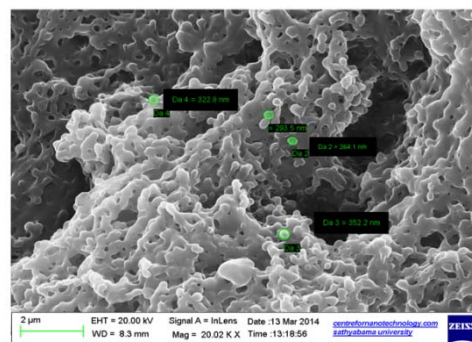


Fig. 7: PHB nanoparticles

CONCLUSION

Bioplastics are gaining importance due to their biocompatible and biodegradable nature which may have a great impact as an alternative for petroleum based plastics. The bacterium isolated from oil soaked cloth was identified as *Bacillus cereus* and was found

to produce PHB up to 47.9% of its dry cell weight. The rate of growth as well as the PHA production increased up to 24 h of incubation which decreased slowly on further incubation. FTIR, GC-MS and NMR analyses clearly confirmed the polymer as PHB. The peaks obtained correlates well with the previous findings. Morphology and crystalline nature of the biogenic PHB have confirmed using FESEM and XRD analyses respectively. The organism seems to be a potential candidate for the biogenic synthesis of polyhydroxybutyrate. Future plan of work includes the production of PHB using agricultural wastes such as sugarcane bagasse, sugarbeet molasses etc. which will make the process cost effective.

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CONFLICT OF INTEREST

Declared None

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