Synthesis and characterization of poly-β-hydroxybutyrate from Bacillus thuringiensis R1

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A poly-β-hydroxybutyrate (PHB) accumulating, Gram positive Bacillus thuringiensis R1 was isolated from the soil samples. The growth of the organism and PHB accumulation in the presence of different carbon sources was studied. While maximum dry cell mass accumulation (3.90 to 4.11g/L) resulted in the presence of glycerol (1% v/v), least biomass formation (1.28g/L) was in the presence of 1%(v/v) lactic acid. Glycerol also supported maximum accumulation of PHB (34.18 to 34.23%) on cell dry mass basis. PHB accumulation in the presence of table sugar (sucrose) and molasses was 28.23 % and 23.06%, respectively. No PHB accumulation was observed in the presence of acetic acid or ethanol. Bacillus thuringiensis R1 cells grown to log phase in the basal medium in the absence of a additional carbon source and then reinoculated into the same medium supplemented with 1% (v/v) glycerol or 1% (w/v) table sugar accumulated 64.10% and 31.36% PHB of the dry cell mass, respectively. The recovered polymer was characterized by NMR, FTIR, GPC, DSC and TGA.

Keywords: Bacillus thuringiensis, β-hydroxybutyric acid, carbon sources, biphasic studies, physical characterization

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Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters and elastomers, which accumulate as cytoplasmic inclusions in certain bacteria during unbalanced growth conditions¹-³, usually characterized by an excess carbon supply and the lack of one or more essential nutrients⁴. About 150 different hydroxyalkanoic acids have been identified as constituents of bacterial polyesters⁵. The first PHA, a homopolymer of poly-β-hydroxybutyrate (PHB), was discovered by Lemoigne in 1925⁶. It is a biodegradable plastic and an elastomer that accumulates in several bacterial species under limiting growth conditions. PHB also acts as a carbon storage compound and a sink for reducing equivalents⁷. PHB and its co-polymers have been industrially produced since 1982 as substitutes for petroleum based plastics. Global environment concerns and solid waste management problems have generated much interest in the development of biodegradable plastics that retain the desired physical and chemical properties of the conventional synthetic plastics. PHAs have found wide ranging applications as biodegradable and biocompatible polymers⁸. One of the major stumbling blocks in large-scale synthesis and wide commercialisation of PHAs is the high production cost compared with the conventional petrochemical derived plastic materials⁹. Studies have shown that the raw material costs (mainly carbon source) contribute most significantly to the overall production cost of the PHAs (up to 50% of the total operating costs)¹⁰. Ralstonia eutropha, Alcaligenes latus and recombinant Escherichia coli harbouring the Ralstonia eutropha PHA biosynthetic genes accumulate PHB in presence of glucose or sucrose¹¹,¹². However, Streptomyces aureofaciens and recombinant E.coli expressing the PHB biosynthetic genes from S. aureofaciens have been shown to accumulate high amounts of PHB in the presence of glycerol¹³-¹⁵. Bacillus thuringiensis, better known for its insecticidal δ-endotoxin, has been reported to accumulate PHB¹⁶. Other Bacillus species e.g., B. mycoides RCJ B-017¹⁷, B. megaterium¹⁸, B. subtilis, B. firmus, B. sphaericus and B. pumilus¹⁶ have also been shown to accumulate polyhydroxyalkanoates in varying amounts. The present study reports synthesis and accumulation of higher amounts of PHB (64.1%) by a B. thuringiensis isolate that is higher than any of the Bacillus species isolates reported earlier. The physical and chemical characterization of the accumulated polymer is also described.

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Materials and Methods

Isolation and Identification

Soil samples were collected from different locations of the National Chemical Laboratory, Pune Campus. One gram of each sample was suspended in sterile deionised water and volume made up to 100 mL. The samples were shaken vigorously for 2 min. The supernatant was serially diluted from $10^1$ to $10^8$ times with 0.9% NaCl and 0.1 mL of the diluted samples was plated on nutrient agar medium plates. The plates were incubated at 37°C for 24-48 h.

Analytic Method for PHB Producing Strain

Each bacterial colony obtained on the nutrient agar medium plates was picked and grown in 5 mL of nutrient broth with shaking for 24 h at 37°C. These cultures were then used as the inoculum (1% v/v) for a 50 mL basal medium containing (w/v): yeast extract (1%), peptone (1%), Na$_2$HPO$_4$ (0.1%), MgSO$_4$ (0.02%) and glycerol (1%v/v) as the carbon source. The cultures were grown for 24-48 h at 37°C with shaking at 150 rpm. After incubation, the cell cultures were centrifuged at 4000×g for 10 min at 15°C. The cell pellet was washed with sterile deionised water. An aliquot of the cell pellet was stained with Nile blue-A and observed for orange fluorescence microscope at an excitation wavelength of 460 nm. One such colony showing orange fluorescence was identified as *B. thuringiensis* R1 by colony morphology, Gram staining and biochemical characteristics.

Culture Conditions

The *B. thuringiensis* R1 cells were grown for 70 h at 37°C with shaking at 150 rpm in a basal medium containing glycerol (1%v/v). Samples were harvested at different time intervals; the O.D. at 600 nm and dry weight was recorded. To study the effect of various carbon sources on the growth of *B. thuringiensis* R1 and PHB accumulation, cells were cultivated for 36 h (stationary phase) in the basal medium supplemented with glycerol, glucose, molasses, table sugar (cane sugar), fructose, maltose, lactose, liquid glucose (byproduct of jaggery industry containing 80% glucose), acetic acid, lactic acid, ethanol and β-hydroxybutyrate. Each carbon source was used at a final concentration of 1% (w/v) or (v/v) in the medium except for β-hydroxybutyrate that was used to a final concentration of 0.50% (w/v) in the medium. For biphasic growth studies the *B. thuringiensis* R1 cells were grown in the basal medium for 36 h, harvested and reinoculated in the basal medium supplemented individually with glycerol (1%v/v), molasses (1%w/v), table sugar (1%w/v) and β-hydroxybutyrate (0.50%w/v). The dry cell mass was used for GC analysis to identify and quantify the accumulated PHA.

Extraction of Polymer from Cells

Modified method of Hahn et al. was used to recover the polymer from *B. thuringiensis* R1 cells. Briefly, the cells were harvested by centrifugation at 2000×g, washed twice with deionized water and freeze-dried under vacuum. The lyophilized cell pellet was shaked for 90 min at 37°C with chloroform and 30% sodium hypochlorite (1:1). The dispersion was centrifuged at 4000×g, at room temperature for 10 min. Lower chloroform phase that contained the solubilised polymer was recovered and the polymer precipitated by the addition of 4 volumes of methanol. The precipitated polymer was washed with acetone, dried and used for physical characterization.

Gas Chromatography Analysis

Acidic propanolysis of dried cell pellet was carried out as described previously. About 100 mg of the dry cell pellet was taken in a tightly sealable vial. Two mL of 1,2-dichloroethane (DCE), 2 mL of acidified propanol (1 vol concentrated HCl + 4 vol n-propanol) and 200 μL of the internal standard (2.0 g benzoic acid in 50 mL n-propanol) were added to the pellet and incubated at 85°C for 4 h. The mixture was shaken from time to time. After cooling to room temperature, a mixture of 2 mL DCE and 2 mL acid propanol was added. The sample was centrifuged at 10,000×g for 5 min. The clear supernatant was collected and an aliquot was injected into the gas chromatograph. GC analysis was performed using a Shimadzu GC 17-A gas chromatograph. A 0.32 mm diameter BP1 capillary column (J&W Scientific Co., USA) of 25 m length was used. The analysis started at 80°C for 5 min followed by a 7°C/min rise in temperature to the final temperature of 200°C. Nitrogen (5 mL/min) was used as the carrier gas. Quantitative evaluation was effected by the peak areas of hydroxybutyl and benzoyl esters formed.

Physical Characterization of the polymer

Scanning Electron Microscopy

PHA sample purified from *B. thuringiensis* R1 cells was sonicated at 20 KHz for 3 cycles of 5 min each. A drop of suspension was dried on a brass stub.
under the IR lamp and later coated with gold using Polaron sputter unit. SEM photographs were taken with a Leica Stereoscan 440 Scanning electron microscope equipped with a Phoenix EDAX attachment.

**1H NMR Spectroscopy**

The $^1$H NMR of the extracted polymer was carried out using Bruker Ac200 at 24°C. The polymer was solubilized in CDCl$_3$. The samples were analysed in 5 mm sample tubes in chloroform-d. The spectra were referenced to internal tetramethylsilane.

**FTIR Analysis**

A polymer sample (2 mg) was thoroughly mixed with KBr (Spectroscopic grade). A 15 mg KBr treated pellet was dried at 100°C for 4 h. FTIR spectrum was taken using a Perkin-Elmer (USA) model 1720 Fourier Transform IR spectrometer.

**Determination and Distribution of Molecular Weight by GPC**

The molecular weight and molecular weight distribution of the polymer was determined by gel-permeation chromatography (GPC) using a Waters 515 pump with four Styragel HR columns. Monodisperse polystyrene and chloroform were used as the molecular weight standard and the mobile phase, respectively. For each analysis, 250 μL of 0.1% (w/v) polymer solution in CHCl$_3$ was injected.

**Differential Scanning Calorimetry**

To determine the morphological state of the polymer, the melt temperature and the enthalpy of fusion were measured using a Perkin-Elmer Differential Scanning Calorimeter (DSC-7). Samples of 10-15 mg were encapsulated in aluminium pans and heated from 0 to 200°C at a rate of 20°C/min. The melting temperature ($T_m$) was taken at the peak of the melting endotherm.

**Thermogravimetric Analysis of PHA**

Thermogravimetric analysis of the polymer sample was analysed using Rheometric TG analyser (Rheometric Scientific, USA). The analysis was carried out under nitrogen flow rate of 15mL/min with scanning rate of 10°C/min. The temperature was increased until weight loss was 20%. After cooling rapidly to room temperature (100°C/min), the residue was recovered. This residue was completely soluble in chloroform.

**Results**

One particular bacterial isolate from the soil samples when stained with Nile blue A and viewed under an excitation wavelength of 460 nm, showed characteristic orange fluorescence, indicating the possible presence of PHA(s) in the cells. Based on biochemical characteristics, the isolate was characterized and identified as the endospore forming, Gram-positive *Bacillus thuringiensis*, and designated as the strain R1 (Table 1).

The *B. thuringiensis* R1 cells grown for 24 h in the basal medium containing glycerol (1%v/v) were harvested. GC analysis of the dried and esterified cells revealed the accumulation of PHB. The cells were then cultured in the presence of glycerol (1%v/v) for 70 h to study growth and PHB accumulation patterns. The pH of the medium dropped from 7.2 to 5.5 during this period. *B. thuringiensis* cells entered stationary phase of growth after 36 h of incubation (Fig. 1a).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Response</th>
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<tbody>
<tr>
<td>Gram staining</td>
<td>Positive</td>
</tr>
<tr>
<td>Spore staining</td>
<td>Positive</td>
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<tr>
<td>Cell shape</td>
<td>Rods</td>
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<tr>
<td>Density</td>
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<td>Elevation</td>
<td>Convex</td>
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<td>Margin</td>
<td>Wavy</td>
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<td>Motility</td>
<td>Negative</td>
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<tr>
<td>Fluorescence and pigments</td>
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<tr>
<td>Indole test</td>
<td>Negative</td>
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<tr>
<td>Methyl red test</td>
<td>Positive</td>
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<tr>
<td>Voges Proskauer test</td>
<td>Negative</td>
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<td>Citrate utilization</td>
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<td>Casein hydrolysis</td>
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<td>Urea hydrolysis</td>
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<td>Catalase test</td>
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<td>Cytochrome oxidase test</td>
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<td>Acid production from Glucose</td>
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<td>Sucrose</td>
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Maximum dry cell biomass (3.90 g/L of the medium) and PHB accumulation (1.4 g/L; 34.18% of the dry cell mass) coincided with the advent of the stationary phase of growth. *B. thuringiensis* cell dry biomass formation and PHB accumulation, however, varied with the carbon source (Fig. 1b). In the presence of molasses, lactose and table sugar it was 3.11, 2.67 and 2.01 g/L, respectively. Least cell growth was observed in the presence of ethanol and lactic acid. Dry cell mass in the presence of monomer β-hydroxybutyric acid (0.5% w/v) was 1.5 g/L. PHB accumulation in the presence of table sugar, molasses and fructose was 28.23%, 23.06% and 19.76% (0.57; 0.72 and 0.34 g/L of media), respectively. PHB accumulation in the presence of lactose was 12.72% (0.34 g/L) and 4.61% (0.06 g/L) in the presence of lactic acid. Only 9.23% (0.13 g/L) PHB accumulated in the presence of glucose in the medium. Acetic acid and ethanol did not support any PHB accumulation. Marginal accumulation of PHB (2.10% on cell dry mass basis; 0.03 g/L) was observed with the incorporation of maltose in the basal medium. PHB accumulation was 52.50% of dry cell mass (0.79 g/L) in the presence of β-hydroxybutyric acid monomer in the medium.

Under biphasic growth conditions, *B. thuringiensis* cell dry weight in the presence of glycerol (1% v/v) increased from 4.11 to 6.11 g/L of the medium and PHB accumulation from 34.23 to 64.10% of dry cell mass (1.41 to 3.91 g/L). When table sugar was used in the biphasic study there was an increase in dry weight from 2.01 to 2.70 g/L and PHB content from 28.23 to 31.36% of dry cell mass (0.57 to 0.85 g/L). There was no increase of PHB content in the biphasic growth of *B. thuringiensis* when either the monomer β-hydroxybutyric acid or molasses were used as the carbon source (Table 2).

GC analysis of the dried and esterified *B. thuringiensis* R1 cells grown for 36 h in the presence of different carbon sources, revealed two peaks. One corresponding to the propyl ester of 3-hydroxybutyrate (3-HB) and the other to the propyl ester of the internal standard benzoic acid (Fig. 2).

PHB granules isolated from the *B. thuringiensis* cells observed under scanning electron microscope showed stable spherical configuration with an average diameter of 5 microns (Fig. 3). The NMR spectra identified the polymer as an isotactic homopolymer (Fig. 4). The spectrum revealed the presence of three groups of signals characteristic of PHB homopolymer. The doublet at 1.3 ppm was attributed to the methyl group coupled to one proton; the doublet of the quadruplet at 2.57 ppm to the methylene group adjacent to an asymmetric carbon atom bearing a single proton and the multiplet at 5.28 ppm to the methyne group. Chloroform-d gave a chemical shift signal at 7.25 ppm. FTIR analysis revealed two absorption bands at 1280 cm⁻¹ and 1735 cm⁻¹ corresponding to C=O and C-O stretching groups, respectively (Fig. 5).

The gel permeation chromatography analysis of the polymer isolated from *B. thuringiensis* R1 cells (Fig. 6) revealed that the polydispersity index (Q) (defined...
as Mn/Mw), number average molecular weight (Mn), weight average molecular weight (Mw) were 1.77, 5.8526×10^4 and 1.0385×10^5, respectively. The melting temperature and the enthalpy of fusion of the polymer were 165.6°C and 84.1 J/g, respectively. The polymer degraded rapidly between 225 and 270°C with a peak at 261°C (Fig. 7).

Fig. 2—Gas chromatograms of the propyl ester of the authentic Polyhydroxybutyrate sample (A) and the _B. thuringiensis_ R1 cells grown in medium containing glycerol (B) Propyl ester of PHB and benzoic acid are represented by peaks 1 and 2, respectively.

Fig. 3—Scanning electron microscopy of the PHB granules obtained from _B. thuringiensis_ R1.

Fig. 4—^1^H NMR spectra of the polymer recovered from _B. thuringiensis_ R1 cells.

Fig. 5—FTIR spectra of polymer purified from _B. thuringiensis_ R1.

Fig. 6—GPC of the polymer purified from _B. thuringiensis_ R1 done. Monodisperse polystyrene and chloroform were used as the molecular weight standard and the mobile phase respectively. For each analysis 250 μL of 0.1% (w/v) polymer solution in CHCl₃ was injected.
Discussion

*B. thuringiensis* better recognised for the production of insecticidal δ-endotoxin has been reported to accumulate PHB16. However, no study has been carried out to gauge the influence of media and culture conditions on the accumulation of PHAs by this microorganism. The present study highlights the accumulation of PHB by *B. thuringiensis* R1. Maximum cell mass formation and the accumulation of the polymer coincided with the beginning of stationary phase of the cell growth cycle. Thereafter, the amount of accumulated PHB declined possibly due to PHB metabolism24. GC analysis of the *B. thuringiensis* R1 cells revealed that the cells supported synthesis of the PHB homopolymer when supplied with a single carbon source. However, glycerol supported maximum cell growth as well as PHB accumulation (except for when the hydroxybutyrate monomer was used). The utilization of glycerol for growth and PHB formation ensures conversion of glycerol to acetyl CoA via either glycolysis or the methylglyoxal pathways13. Some of the *Bacillus* species have been reported to accumulate 6-36% PHB of the cell dry mass16. PHB accumulation of 64.1% in the biphasic growth cycle of *B. thuringiensis* R1 cells is the highest recorded for this bacterium so far. This indicates that while the first phase is used for the development of the biomass, the second phase in the presence of glycerol is preferentially utilized for PHB production by the cells. Glycerol, which is the by-product of soap industry, can be utilized as the carbon source for efficient PHB production by the *B. thuringiensis* R1 cells. Table sugar and molasses being cheap sources from the massive sugar industry in India may also be used for cost effective production of PHB on a large-scale. This is the first report of utilization of glycerol, table sugar or molasses for the production of PHB by *B. thuringiensis* cells. Borah *et al*17 reported the use of sucrose as the cheaper source for the production of PHB by *B. mycoides* RLJ B-017. Similarly, *B. Jma*25 (25-35%) and *B. megaterium*18 (40.8%) accumulate PHB during fermentation with molasses. *B. megaterium*18 has also been reported to accumulate PHB in the presence of glucose (39.9%). Belma *et al*16 have reported PHB accumulation of 8.0 and 7.5% in *B. thuringiensis* strains D1 and D2, respectively in glucose containing medium. In the present study, glucose did support high accumulation of PHB (9.2%). This may be possibly the result of medium acidosis since the cell mass formation was also low. It is also documented that glucose in the medium represses the expression of phosphotransbutyrylase (Ptb) gene that is needed for PHB production26. Glucose is metabolized through the EMP pathway to yield pyruvate and acetate as the main products. Acetate is partially converted into PHB, which is consumed during sporula-
production in *Bacillus* sp. 27. Although acetate is a precursor in acetyl-CoA synthesis 1 it did not support PHB accumulation by the *B. thuringiensis* strain R1. Though there was increased accumulation of PHB (52.5%) with the use of β-hydroxybutyrate (sodium salt) as the carbon source in the medium, it is not feasible to use the monomer as a carbon source owing to its high cost. Growth of the *B. thuringiensis* culture in two phases resulted in the increase of PHB production when glycerol or table sugar was used as the carbon source. This indicates that the fully grown culture of *B. thuringiensis* could utilize the carbon source supplied in the second phase, essentially for the accumulation of PHB. This biphasic growth for PHB accumulation may be used as a strategy for increased PHB production on a commercial scale in *B. thuringiensis* as reported in other bacteria.

The NMR signal multiplicity by a proton as a quadruplet or octet in case of protons of CH2 group was obtained due to proton coupling in isotactic form unlike in syndiotactic where duplet signal is obtained due to coupling. Similar spectral signatures have been reported for PHB isolated from *B. cereus* 28 and *B. circulans* 22. Rozsa et al 22 have shown that the FTIR absorption band at about 1730 cm⁻¹ is a characteristic of carbonyl group and a band at about 1280-1053 cm⁻¹ characterizes the valence vibration of the carbonyl group. These are characteristics of the polyhydroxybutyrate.

The high enthalpy of fusion (84.1 J/g) suggests high crystalline nature of the recovered PHB that was calculated to be of 60-65% assuming the enthalpy of fusion of 100% crystalline sample to be 146 J/g. The melting temperature of PHB (165.6°C) in the present study was slightly lower than reported for PHB from *B. cereus* 28 (170°C) and *B. circulans* 22 (173°C). The difference between the melting temperature and the decomposition temperature (261°C) was high enough to facilitate processing of the polymer.

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**References**


