Pak. J. Bot., 48(1): 349-356, 2016.

PRODUCTION AND CHARACTERIZATION OF A THERMOSTABLE BIOPLASTIC (POLY-ß-HYDROXYBUTYRATE) FROM *BACILLUS CEREUS* NRRL-B-3711

ASAD-UR-REHMAN*, ALIA ASLAM, RUSHDA MASOOD, MUHAMMAD NAUMAN AFTAB, RABIA AJMAL AND IKRAM-UL-HAQ

Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan.
**Corresponding author's email: drasadgcu@gmail.com; Tel: +923314249096*

Abstract

The poly-β-hydroxybutyrate (PHB) is a thermostable and biocompatible polyester produced by several bacteria under unbalanced nutritional conditions. Among Gram-positive bacteria, which are not well exploited for PHB production on industrial scale, *Bacillus* appears to be a prospective candidate due to its excellent biopolymer yield and less rigorous fermentation conditions. Batch culture fermentation was carried out for PHB production. The *Bacillus cereus* NRRL-B-3711 was the most efficient producer of PHB out of five *Bacillus* species. The optimized production was achieved with 2% glucose, 37°C, pH value of 8 and ammonium sulfate (2.5 g/L) as a nitrogen source. Carbon to nitrogen ratio of 10 significantly affects the PHB accumulation. The selected specie was able to accumulate PHB up to 56% (14.2 ±0.07 g/L) on dry cell weight basis after optimization. This corresponds to a 1.87 folds increase in the production. The optical microscopy showed extremely flat surface of bioplastic thin film indicating the brittle failure of PHB under tensile loading. The FTIR analysis revealed C=O and –CH groups, with thermal properties e.g. *Tg;* 2°C, *Tc*; 54°C, *Tm;*162°C and % crystallinity of 51.3 by differential scanning calorimeter (DSC), thus confirming the presence of PHB bioplastic and enhancing its industrial applications.The results surpassed those reported in the literature for PHB production.

Key words: Poly β-hydroxybutyrate, Thermostable, Biocompatible, Fourier transform infrared (FTIR) spectroscope, Differential scanning calorimeter (DSC).

Introduction

Synthetic plastics derived from petroleum find significant importance in every sphere of human life, having a wide range of applications in industrial and consumer products. At the same time petroleum-based plastics are becoming more expensive as oil prices continue to increase (Khare & Deshmukh, 2006). Moreover, due to the plastic's native persistency and its resistance to degradation, an environmentally brutal waste problem is being created. Direct discard in nature causes landfill disasters because such conventional plastics persist for many decades. On the other side, disposal of plastic by incineration can also cause severe air pollution problems because chemical additives added during production of the plastic generate acidic gases, dioxins, carbon dioxide and many other toxic gases. Therefore, living "green" has become a popular trend in the last twenty years, and reducing oil consumption remains an important goal for the sustainably-minded today (Contreras *et al*., 2013). Consequently, it is required to employ sustainable and eco-friendly biodegradable plastics instead of conventional petroleum-derived.

During recent years, a range of bioplastics have been presented to use for various purposes, which are not only attuned with human lifestyle but also are friendly to the environment (Eversloh *et al*., 2001). Polyhydroxyalkanoates (PHAs) are considered to be the most promising class of biopolymers with diverse applications. Polyhydroxybutyrate (PHB) is a biodegradable small chain length (scl) PHA. The intracellular granules of PHB are synthesizd and accumulated in more than 300 genera of bacteria encircling Gram-positive (*Actinomycetes*, *Bacillus* etc) as well as Gram-negative (*Alcaligenes*, *Mthylobacterium*, *Pseudomonas*etc) species (Steinbuchel *et al*., 1992). Although Gram-negative bacteria are the only commercial source of PHB at present (Valappil *et al*., 2007b), the Gram positive bacteria are focused now-a-days for the production of PHB. Several properties including petite generation period, the ability to utilize inexpensive carbon sources for PHB production as the presence of both amylase and protease indicates that it even uses food wastes as substrate, and the absence of endotoxins (LPS), which in other Gram negative bacteria co-purify with the biopolymer and cause immunogenic reactions (Law & Slepecky, 1992; Valappil *et al*., 2007a), are constructive traits which justify demanding exploration into their PHB production capabilities. Among Gram-positive bacteria, *Bacillus* appears to be a prospective candidate for the PHB accumulation due to its excellent biopolymer yield and less rigorous fermentation conditions. Moreover, the novel PHB synthase identified from *Bacillus* has the ability to integrate both short chain length (scl) and medium chain length (mcl) PHA, showing that the genus can be a strong producer of new and known PHAs with diverse monomeric composition (Thirumala *et al*., 2010).

Physical properties of PHB are similar to synthetic polymer; therefore, it is feasible to produce PHB at industrial scale. The elevated production cost of biopolymer restricts its broad range of popularization as conventional plastics. Therefore, cheap substrate, improved cultivation strategies and uncomplicated downstream processing methods are necessitated for cost reduction. Optimization of the fermentation parameters (carbon and nitrogen source, temperature, pH, time and carbon to nitrogen ratio) are, therefore, significant to lessen the cost because they effect the metabolism of microorganisms.

In current study, the potential *Bacillus* specie for PHB synthesis was screened out of five different species of *Bacillus*. The composition of fermentation medium and experimental conditions were optimized to develop the biomaterial through biotechnology. The carbon to nitrogen ratio is the most crucial factor which influences the accumulation rate of PHB. To the best of our knowledge, the current report is the first detailed study on the effect of carbon to nitrogen ratio on PHB accumulation by *Bacillus cereus* and its topological feature which is a vital trait for its domestic and industrial applications. An important and unique observation e.g. sporulation phenomenon was also studied in context of its effect on PHB accumulation. The thermal properties (T_c, T_m, T_g and % crystallinity) of extracted biopolymer were studied by DSC which revealed stability at high temperature thus directly influencing its commercial aspects. The functional groups of the extracted bioplastic along with determination of crystallinity index was carried out using FTIR.

Materials and Methods

Chemicals: All the chemicals were of analytical grade and purchased from Sigma chemicals (USA), Fisher Scientific (UK), Merck (Germany) and Acros Organics (Belgium).

Microorganisms: Five different bacterial species viz., *Bacillus subtilis*NRRL-B-941, *Bacillus licheniformis* NRRL-B-1001, *Bacillus cereus* NRRL-B-3711, *Bacillus megaterium*NRRL-B-3712 *and Bacillus thuringiensis*798 obtained from the stock culture of Institute of Industrial Biotechnology (IIB), GC University Lahore, were used in the present study. All cultures were maintained on nutrient agar slopes at 4 °C and sub-cultured every 2 weeks.

Qualitative screening of PHB producing *Bacillus* species: The *Bacillus* species were qualitatively evaluated for PHB production followed by the viable colony method of screening using Sudan Black dye (Liu *et al*., 1998). For rapid screening of PHB producing microorganisms, nutrient agar medium was supplemented with 1 % glucose and sterilized. The medium was aseptically poured into sterile Petri plates and allowed for solidification. The plates were divided into 5 equal parts and in each part, a *Bacillus* specie was spotted. The plates were then incubated at 30^{o}C for 24 hours. Ethanolic solution of Sudan Black dye was spread over the colonies and the plates were kept undisturbed for 30 minutes at room temperature. The plates were then washed with ethanol (96%) to remove the excess stain from the colonies. The dark blue colored colonies were taken as positive for PHB accumulation.

Seed culture preparation: Nutrient agar slants were used to prepare the seed cultures. A loopful of each bacterial culture was transferred into separate 250 ml Erlenmeyer flask containing 25 ml sterile nutrient broth, under aseptic conditions. The flasks were then incubated at 37^{o}C for 24 h at 200 rpm in a rotary shaking incubator (VS-8480, Vision Scientific Co. Ltd, Japan).

Fermentation technique: Submerged fermentation technique was employed in the present investigation. The fermentation medium for the production of PHB was prepared by adding separately sterilized carbon source and trace elements solution to the basal medium. The fermentation medium adopted from Bora (2012) was used for the bacterial growth and PHB production (Bora, 2012). The composition of final fermentation medium was as follows (g/L); glucose, 20; $(NH_4)_2SO_4$, 2.5; KH_2PO_4, 1.5; Na_2HPO_4, 3.5; $MgSO_4.7H_2O$, 0.2; trace element solution 1ml/L. The composition of trace element solution was as follows (g/L); $ZnSO_4.7H_2O$, 0.246; $MnSO_4.H_2O$, 0.169; $CoCl_2.6H_2O$, 0.237; H_3BO_3, 0.061; $CuSO_4.5H_2O$, 0,249; $NiCl_2.6H_2O$, 0.237; $NaMO_4.2H_2O$, 0.442; $CaCl_2.2H_2O$, 0.147; $FeSO_4.7H_2O$, 0.278 and KI, 0.166). Fifty milliliters of the fermentation medium was poured into each of 250 ml Erlenmeyer flasks which were cotton plugged and sterilized in an autoclave (KT-40L, ALP Co. Ltd., Tokyo, Japan), at 121°C,for 20 min. The flasks were cooled at room temperature and inoculated with 2.0 ml of the pre-culture, under aseptic conditions. The flasks were then incubated in a rotary shaking incubator (VS-8480, Vision Scientific Co. Ltd, Japan) at 200 rpm, 37^{o}C for 48 h. To test the effect of different carbon sources on PHB production, glucose was replaced by other carbon sources. All experiments were conducted in triplicates. At the end of incubation period, PHB, cell dry weight and residual glucose were estimated.

Analytical Techniques

Qualitative analysis of endospores: During polymer production, samples of the screened bacteria were checked for the formation of endospores using the Schaeffer–Fulton method (Doetsch, 1981). The samples were air dried on a glass slide, heat fixed and covered with filter paper. The slide was then saturated with aqueous solution of malachite green dye (0.5% w/v) and placed over a boiling water bath for 5 min. The filter paper was then removed and the slide was washed in distilled water. Counter staining of the slide was carried out with safranine (2.5 g of safranine in 100 ml of 98% ethanol) for 30 sec, followed by washing under tap water and blot drying using a bsorbent paper. The preparation was then examined under the light microscope (XSZ-107BN China) using an oil immersion objective (100×).

Determination of poly-β-hydroxybutyrate: Dry cell weight (DCW) was evaluated gravimetrically. A known volume of culture sample was centrifuged (D-78532, Hettich Zentrifugen EBA 20, Tuttligen, Germany) at 8,000 × g for 15 min. The supernatant was used for further analysis while the cell pellet was washed twice with distilled water and dried to constant weight in a hot air oven. The residual glucose in the fermentation medium was determined according to Miller (1959).

The crotonic acid assay was used for the assessment of PHB amount as reported by Aslim *et al.* (2012) and adopted by several other workers (Singh *et al*., 2012). Dry cell pellet was incubated at 60°C for 1 h with 5 ml of 6% sodium hypochlorite to break the cell wall of bacteria. After the incubation, solution containing biomass and sodium hypochlorite turned white, it was considered that all the cellular biomass was being digested. The PHB granules were then collected by centrifugation at 10,000 × g for 15 min. Cell lipids and impurities (except PHB) were extracted by washing with distilled water, acetone and absolute ethanol, sequentially. The pellet thus obtained was dissolved in 5 ml of boiling chloroform. The chloroform was evaporated to obtain PHB at room temperature. By adding 5 ml of 98% sulphuric acid and boiling for 1h, PHB crystals were converted into crotonic acid. Crotonic acid absorbs ultraviolet light. The absorbance of the solution was measured at 235 nm in a UV/Vis spectrophotometer (5000 Irmeco GmbH, D-2149 Gee Germany) against a sulphuric acid blank. The amount of PHB was calculated using standard curve of crotonic acid. All analysis were carried out in three replicates.

Characterization of PHB

Optical microscopy: A thin film of PHB was prepared by solvent casting method (Thirumala *et al*., 2010). A 2% solution of PHB was prepared in chloroform. An even, plane and scuff less glass plate was selected for film casting. The plate was placed on a smooth and leveled surface to obtain a film of the even thickness. A spirit leveler was used to echelon the plates. The chloroform solution of 20-80 ml was poured in the glass plate and left in a place without air turbulence for about 4 h at room temperature. Care was taken not to disturb the plates during drying. After drying the plate, the film was peeled out of the plate. The outer layer of the casted biopolymer thin film was studied by optical microscope (Olumpus Corporation, Japan) at different magnification powers (100, 200 and 500x) (Fig. 7).

Fourier transform-infrared spectrometer (FTIR) analysis: The extracted bioplastic was analyzed on Fisher scientific FT/IR spectrometer (FT/IR-4100 typeA, Fisher scientific, UK limited) range of 400- 4000 /cm. The KBr discs were prepared by mixing PHB and research grade potassium bromide at a ratio of 1:100 (Kemp, 1989). Moisture content was removed by pressing in suction pump for 15 min to obtain a translucent KBr pellet. IR spectra were recorded with 4/cm resolution.

Differential scanning calorimetric (DSC) analysis: The thermal properties of biopolymer i.e. glass transition temperature, (*Tg*); crystallization temperature, (*Tc*); melting temperature, (*Tm*); enthalpy of fusion and crystallinity (%) were studied by using differential scanning calorimeter, (DSC) (TA series Q 600). The amount of polymer used for analysis ranged from 0.8-1.0 mg encapsulated in standard aluminium pans. Smaller amount of sample was used in order to ensure temperature uniformity and good reproducibility. All experiments were carried out using inert nitrogen. The samples were heated and temperature was increased at a rate of 10°C /min.

Statistical analysis: The statistical analysis was conducted after Snedecor and Cochran (1989). The SPSS v.17.0 was employed for this purpose and the data varied significantly at $p \leq 0.05$.

Results and Discussion

Qualitative and quantitative screening of *Bacillus* species: Different bacterial species such as *Bacillus subtilis* NRR-B-941, *Bacillus licheniformis*-B-NRRL 1001, *Bacillus cereus* NRRL-B-3711, *Bacillus megaterium* NRRL-B-3712 *and Bacillus thuringiensis* 798 were screened on the basis of qualitative tests (Sudan black B staining) and quantitative method employing submerged fermentation technique. The Sudan black B staining was used as first line of qualitative observation of PHB production for the bacterial species as also suggested by certain workers (Patel *et al*., 2011). All species gave positive results with bluish purple color of PHB granules present in pink cytoplasm. Followed by such tests, the potential specie for maximum PHB production among five *Bacillus* species was screened out using submerged fermentation. For this purpose, all the strains were grown in nitrogen deficient fermentation medium with glucose as carbon source, because it is easily available, rich in carbon and almost all bacteria utilize it efficiently for their growth (Patel *et al*., 2011). Among these, the maximum PHB production of 47% (7.5±0.04 g/L) on the basis of DCW content was observed with *Bacillus cereus* NRRL-B-3711, while the minimum PHB accumulation of 34% (w/w) (1.04±0.03 g/L) of DCW content was obtained with *Bacillus thurengiensis* (Fig. 1a). Therefore, *Bacillus cereus* NRRL-B-3711 was found to be the most potential strain for PHB synthesis.

Screening of effective carbon sources for PHB production: Three functions are basically performed by carbon source within the microorganism: biomass production, cell continuance and PHB polymerization (Thirumala *et al*., 2010). When tested for various carbon sources, the strain exhibited nutritional versatility with respect to diverge growth and maximum intensity of PHB production. In current work, different carbon sources viz., glucose, mannitol, mannose, glycerol, lactose, sucrose and fructose were used as substrates and tested for their effects on PHB accumulation by *B. cereus* (Fig. 1b). In current study *B. cereus* NRRL-B-3711 gave highest PHB yield of 48.28% (7.59±0.09 g/L) on DCW basis using glucose as a sole carbon and energy source. All the carbon sources were utilized by the bacterial strain. Lopez *et al.* (2012) illustrated production and accumulation of maximum PHB of 29.2% on the basis of DCW by *Bacillus* sp. when grown on glucose. The same strain gave 45.83% on DCW basis with glycerol and also a significant yield of 47.44% on DCW basis with fructose as a carbon source. Therefore, this study was momentous, since it identified a strain which was proficient of producing PHB not only from glucose but also from substrates such as glycerol and fructose.

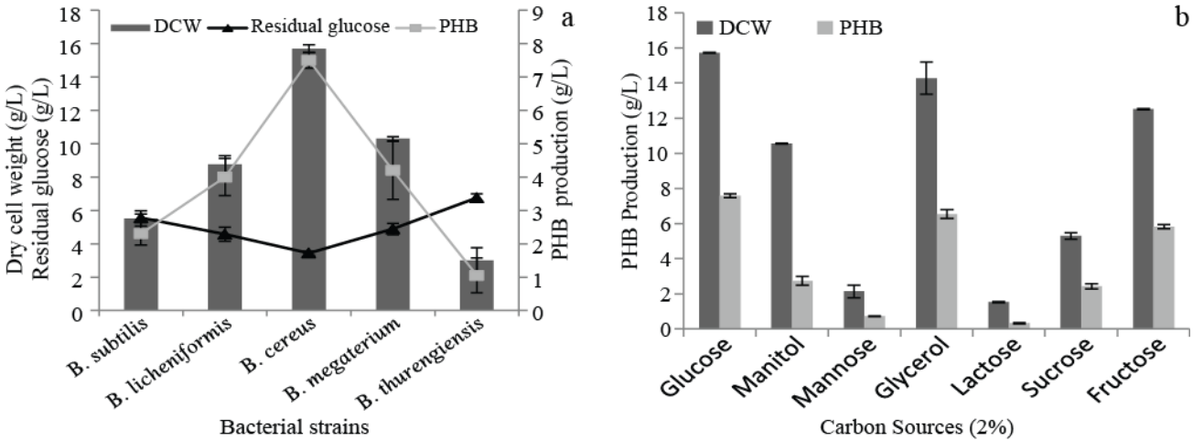


Fig. 1. Screening of various *Bacillus* species and evaluation of carbon sources for PHB production by *Bacillus cereus* NRRL-B-3711 (a) Screening of bacterial species using glucose as a carbon source under submerged fermentation. (b) Evaluation of different carbon sources. Each Y bar indicates the standard deviation among three parallel replicates.

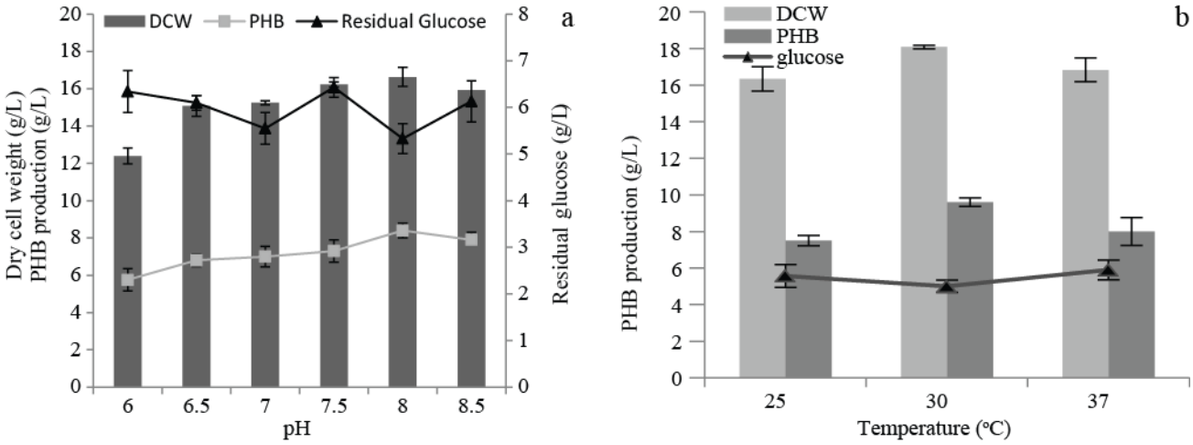


Fig. 2. Effect of pH and temperature on PHB production by *Bacillus cereus* NRRL-B-3711(a) effect of pH (b) effect of temperature. Each Y bar indicates the standard deviation among three parallel replicates.

Effect of pH: The effect of a range of pH values from 6 to 8.5 were observed for maximum PHB accumulation by *Bacillus cereus* NRRL-B-3711.The pH is a crucial factor for the production of metabolites and growth of microorganisms as it influence the metabolic activities and also the synthesis of the PHB (Bora, 2012). According to the results of current study, the maximum PHB production of 50% (8.4±0.06 g/L) on the basis of DCW was reported at pH 8 which is optimum for *Bacillus* species. Residual glucose was minimum at pH 8 which indicated the efficiency of organism to utilize carbon source for its growth and PHB accumulation (Fig. 2a). The pH below 8 significantly lowered the accumulation of PHB (Valappil *et al*., 2007b). Hashimoto *et al.* (1993) and Senthil & Prabakaran (2006) reported the similar outcomes for maximum PHB production. This might be due to the fact that pH above and below the optimal level affects the degenerative enzymes of the biopolymer producing bacteria. By increasing pH from optimal level degenerative enzymes trigger the breakdown of biopolymer and, consequently, the PHB consumption starts at the same rate as it is produced (Nakata, 1963). In current study, the pH of the culture was checked after the fermentation which was changed (4.8-5.5) due to the metabolic activities of the microorganism. This surveillance was consistent to a previous report on a *Bacillus* sp. where lower pH conditions have been reported to inhibit consumption of PHB as well as spore formation in *B. cereus* (Kominek & Halvorson, 1965).

Effect of temperature: The effect of different temperatures such as 25, 30 and 37°C was studied for the PHB production by *Bacillus cereus* NRRL-B-3711. Incubation temperature plays a pivotal role in the metabolic process of an organism (Ramadas *et al*., 2012). The fermentation temperature in the range of 28-45ºC is superlative for the production of PHB (Fig. 2b). Optimum temperature for the production of PHB corresponds to the growth temperature of particular microorganism. Commonly, PHB is produced by *Bacillus* sp. in the range of 30-38°C (Thirumala *et al*., 2010). The maximum PHB production of 53% (9.6±0.06 g/L) on the basis of DCW was obtained at 30ºC. As the temperature increased, the rate of physiological processes also increased which enhanced the growth of microorganism to a certain limit, after that it started decreasing. When the temperature was further increased up to 37ºC the PHB yield was declined i.e. 47% of DCW. These results are in agreement with Arthi and Ramana (2011).

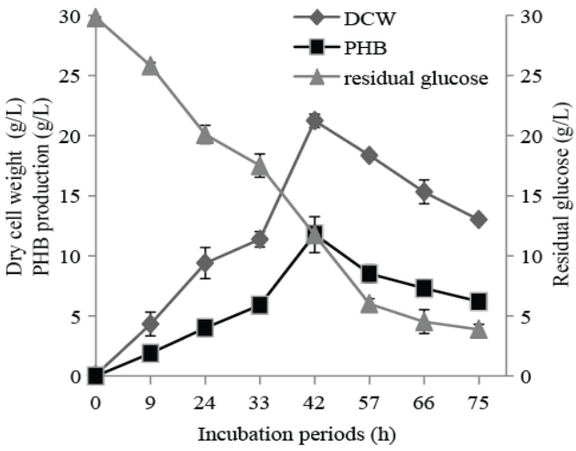


Fig. 3. Effect of incubation period on PHB production by *Bacillus cereus* NRRL-B-3711. Each Y bar indicates the standard deviation among three parallel replicates.

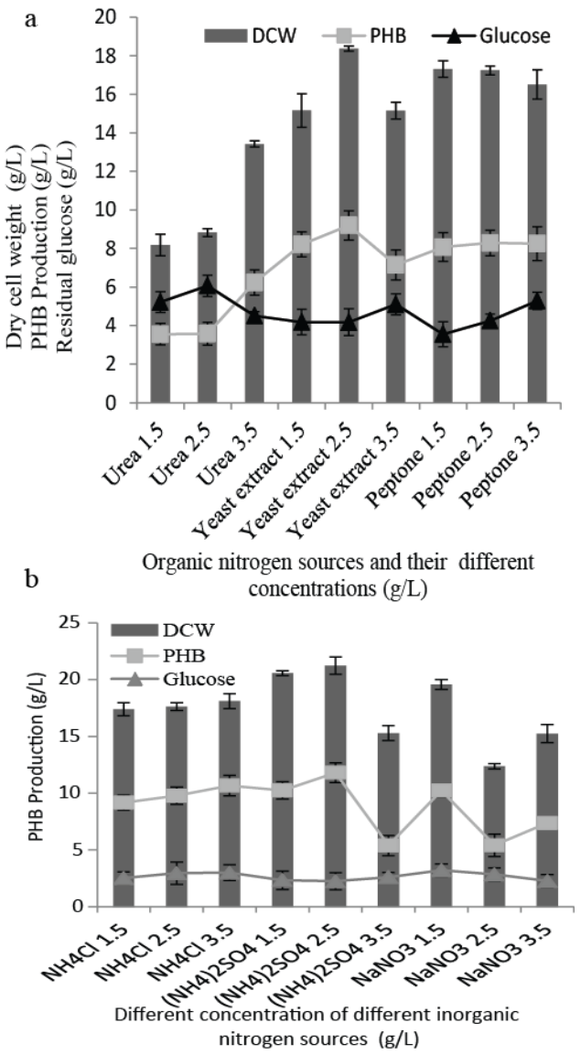


Fig. 4. Effect of organic and inorganic nitrogen sources and their different concentrations on PHB production by *Bacillus cereus* NRRL-B-371. (a) Effect of organic nitrogen sources (b) Effect of inorganic nitrogen sources. Each Y bar indicates the standard deviation among three parallel replicates.

Effect of incubation period: The influence of incubation period ranging from 0 to 75 h was studied for the optimization of PHB production process. Results are depicted in Fig. 3. It has been reported that bacteria able to produce PHB are divided into two categories. The first category involves those which produce PHB during stationary phase when N, P, Mg and oxygen are limited and carbon source is in surplus while, second category involves PHB accumulation during the growth phase (Flora *et al*., 2010). The *Bacillus cereus* belongs to first category. The physiological features of the microorganism are imperative for the production of bioplastic. In this study, the fermentation medium was incubated for different time intervals (0-75 h) (Fig. 3). The PHB production at 0, 9 and 24 h was minimum. It was due to the fact that the culture was in acclimatization phase. The cell mass increased steadily, leading to a maximum cell density at 42 h of incubation followed by a gradual decline. The pH was decreased from its preliminary value of 8 to a minimum of 4.8. The termination of logarithmic growth overlaps with the approach of minimum pH value and rapid consumption of glucose. Diminution in glucose concentration was an indication that microorganism used it as a substrate and underwent active metabolism. The PHB produced rapidly during the stationary phase and a maximum yield e.g. 55% (11.75±0.05 g/L) on the basis of DCW was observed at 42 h of growth. Another unique and important observation in this study is that once maximum PHB concentration was achieved, the PHB concentration started decreasing in order to be utilized for sporulation. The extent of sporulation was tested at regular intervals in this study using Schaeffer-Fulton method and the results were always positive (Fig. 6). This observation is in contrast to the report of Valappil *et al.* (2007b). After the optimal incubation period, the PHB production decreased. It was due to presence of bacteria in its decline phase due to decreased amount of nutrient supplement, accumulation of metabolites, toxins and inhibitors as also reported by other workers (Valappil *et al*., 2007b). During the decline phase when bacteria undergo scarcity of food due to the absence of energy supply, they release definite PHB depolymerase which hydrolyze the polymer to water soluble monomers or oligomeric esters. The hydrolysed products are taken up by the cells and metabolized (Kumaravel *et al*., 2010). Similar results were shown by Valappil *et al.* (2007b) which demonstrated the maximum PHB accumulation at 48 h of incubation time.

Effect of organic and inorganic nitrogen sources: Both organic and inorganic nitrogen sources crucially effect the PHB production. In the fermentation medium both types of nitrogen sources were supplemented for enhancing PHB accumulation. Yeast extract (2.5 g/L) provided maximum production of 50% (9.2±0.07 g/L) on the basis of DCW among all organic nitrogen sources (Fig. 4a). The effect of different inorganic sources like ammonium sulfate, ammonium chloride and sodium nitrate on PHB bioproduction was also investigated in our study. Among all of them, ammonium sulfate was best for PHB synthesis (Fig. 4b). Highest biopolymer production of 55% (11.8±0.04 g/L) on the basis of DCW was obtained by employing 2.5 g/L ammonium sulfate in the medium. It might be due to the fact that yeast extract contains plenty of mineral ions which are complex, so that bacteria could not use it efficiently for the maximum PHB accumulation. The same results were reported by Halami (2008) which indicated that the maximal growth of bacteria occurs in the presence of inorganic nitrogen sources (Gouda *et al*., 2011).

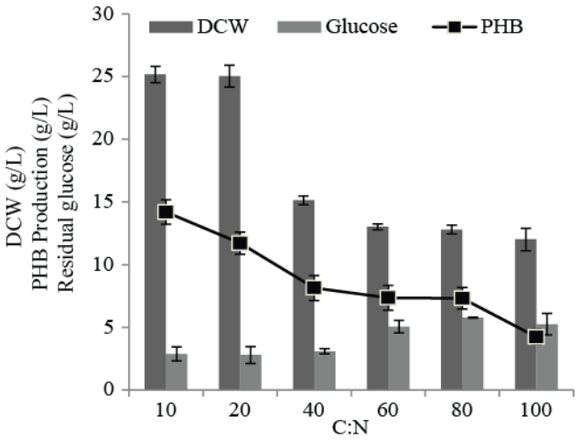


Fig. 5. Effect of carbon to nitrogen ratio on PHB production by *Bacillus cereus* NRRL-B-3711. Each Y bar indicates the standard deviation among three parallel replicates.

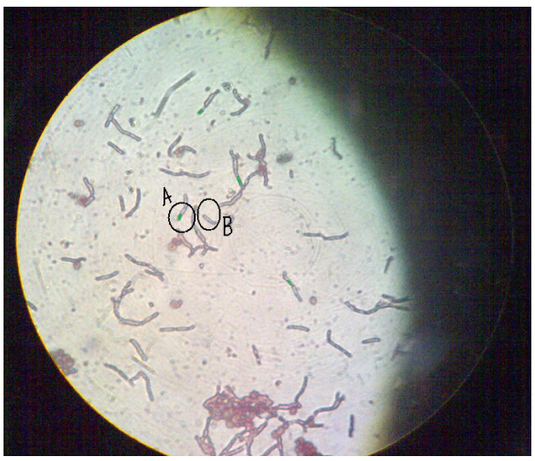


Fig. 6. Spore staining (a) the endospore with green color and (b) the pink colored vegetative cell.

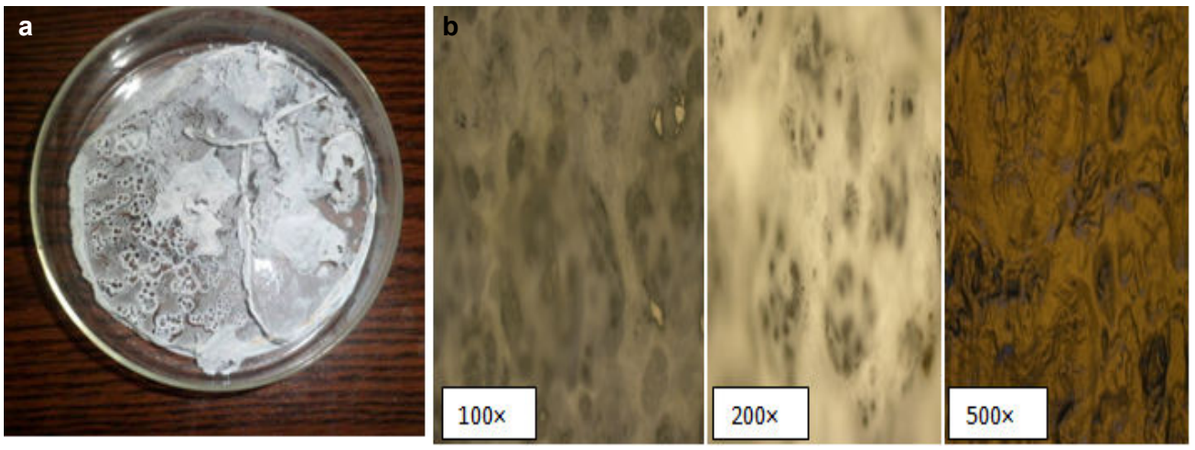


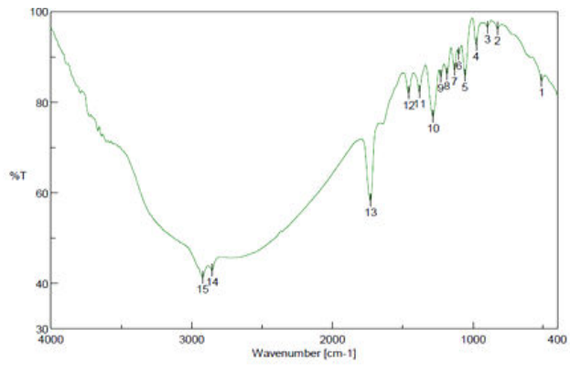
Fig. 7. Physical appearance and optical microscopy of PHB Bioplastic (a) Appearance of recovered PHB biopolymer films. (b) Optical topological photomicrographs of the extracted PHB film at different magnification powers (100×, 200× and 500×).

Effect of carbon to nitrogen ratio: Carbon to nitrogen ratio is another most critical factor which significantly influences the intracellular accumulation of the bioplastic (Grothe *et al*., 1999). It has been suggested that most suitable C:N for the PHB accumulation is likely to diverge with the bacterial strain and sort of carbon and nitrogen supply (Tsuge *et al*., 1999). In current study, a range of carbon to nitrogen ratio was studied by varying nitrogen source (ammonium sulfate) and keeping carbon source (glucose) constant. The range included 10, 20, 40, 60, 80 and 100 (Fig. 5). The maximum PHB yield of 56% (14.2 ±0.07 g/L) on the basis of DCW was obtained at C:N of 10 which was greater than PHB amount of 46% (11.7±0.06 g/L) on the basis of DCW obtained at C:N of 20. The PHB production was decreased at higher C:N of 40, 60, 80 and 100 with lowest observed at 100, which might be due to the fact that at higher carbon to nitrogen ratios *Bacillus* strain underwent sporulation process, in which organism entered in dormant condition with the formation of heat stable cells (Fig. 6). During this phase organism starts to utilize its energy reserves (PHB granules), hence lowering the bioplastic accumulation. The study conducted by Kulpreecha *et al*. (2009) supported the present results, according to which *Bacillus megaterium* showed 1.23 fold increase in dry cell weight and PHB accumulation when carbon to nitrogen ratio was shifted from 25 to 10.In present study 1.21fold (14.2 ±0.07 g/L) increase in PHB occurred when carbon to nitrogen ratio was shifted from 20 to 10. Similar results were exhibited by Arthi & Ramana (2011).

The optical surface of neat PHB film was whitish and without cracks. The topological studies showed extremely flat surface indicating the brittle failure of PHB under tensile loading. Such texture indicates point out no apparent plastic deformation before fracture (Fig. 7a). The microscopy specifies homogeneous matrix, without the presence of remnants of starch granules in the extracted PHB bioplastic thin film (Fig. 7b).

Fourier transform-infrared spectrometer (FTIR) analysis: The FTIR analysis of recovered polymer revealed absorption bands at 1728/cm corresponding to ester carbonyl group (C=O) and at 1284/cm corresponding to the –CH group, characteristic of poly 3-

hydroxybutyrate (Valappil *et al*., 2007a), confirming the production of a scl-PHAs (Fig. 8). The band at 1728/cm is characteristic of crystalline phase and the bands at 2853 and 2922/cm corresponds to aliphatic C-H group of polymer backbone (Steinbuchel *et al*., 1992). The band formed at 1457/cm indicates asymmetrical deformation of the C-H bond in CH_2 groups and the band at 1380/cm corresponds to symmetric wagging of CH_3 groups. The band at 1227/cm is the conformational band of helical chains whereas the bands at 1185 and 1132/cm are characteristic of asymmetric and symmetric stretching vibration of the C-O-C group, respectively. The band at 1380 and 1457/cm indicated CH_2-S and –CH_2The remaining bands located at 1,000- 1,300 /cm assigned to the stretching of the C-O bond of the ester group. The band at 1457/cm is reference band and is insensitive to the degree of crystallinity, whereas that at 1227/cm has the largest difference between crystalline and amorphous states. Therefore, a crystallinity index is the ratios of the band's intensities at 1227/cm and 1457/cm, which is 0.84 for extracted PHB bioplastic.



Result of Peak Picking

No.	Position	Intensity	No.	Position	Intensity
1	512.972	84.7933	2	826.348	96.2418
3	896.737	96.577	4	977.733	92.7639
5	1055.84	85.8653	6	1100.19	90.6131
7	1132.01	87.1536	8	1185.04	86.2528
9	1227.47	85.681	10	1284.36	76.8933
11	1380.78	82.277	12	1457.92	82.0262
13	1728.87	58.3009	14	2853.17	42.933
15	2922.59	41.2883			

Fig. 8. FTIR spectrum for isolated PHB bioplastic.

Differential scanning calorimetric (DSC) analysis: Thermal properties of biopolymer are important which provide information about thermal and oxidative stability of biopolymer, phase and phase changes happening in polymer and their shelf life under specific conditions. Studies on the DSC gave details about the melting temperature of PHB as it is partially crystalline biopolymer so their thermal properties are expressed in terms of glass to rubber transition temperature (*Tg*) of the amorphous phase and the melting temperature (*Tm*) of the crystalline phase. There was an exotherm near 54^{o}C, which is considered to be crystallization temperature (*Tc*) and also an endotherm near 162^{o}C.The glass transition temperature (*Tg*) is a temperature at which the polymer losses its glass like appearance and become rubbery (more flexible) in nature. In current study, *Tg* of 2°C of extracted biopolymer is supported by literature (Thirumala *et al*., 2010).

Melting temperature of the current study is much greater than the *Tm* of 120^{o}C reported in literature (Singh *et al*., 2012). As these temperatures set the "window of processability" which is the range of various processing techniques e.ga melt extrusion or blowing of plastic films etc. So this study is significant in the thermal characterization of PHB bioplastic. These fluctuations in the melting temperature are pertained to the different molecular weights of biopolymer which increase or decrease during recovery phase involving excessive washing with different organic solvents (Singh *et al*., 2012). The % crystallinity of the bioplastic was estimated by heat of fusion of PHB from the thermograph, which was 51.3%, indicating the extracted bioplasticis crystalline, which is in agreement with literature (Thirumala *et al*., 2010).

Conclusion

In current study, a 1.87 folds increase in bioplastic (PHB) accumulation was achieved by selecting an appropriate composition of the fermentation medium and the experimental (physical) conditions. The PHB accumulation soared concomitantly with the biomass. The results disclose imperative consequences of the carbon, nitrogen sources and C:N ratio to produce green plastic through biotechnology. The topological studies give idea about surface texture and roughness which directly influence the applications of the extracted bioplastic. An insight into the thermal stability and degradation has been provided as well with promising potential for industrial exploitation.

Acknowledgements

The authors express their gratitude to the Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan for the laboratory facilities and financial support for this research work. Authors also acknowledge the University of Engineering and technology (UET) Lahore, for providing facilities to characterize the biopolymer.

References

Aarthi, N. and K.V. Ramana. 2011. Identification and characterization of Polyhydroxybutyrate producing *Bacillus cereus* and *Bacillus mycoides* strains. *Int. J. Env. Sci.,* 1: 976-4402.

Aslim, B., N.S. Yuksekdag and Y. Beyatli. 2012. Determination of PHB growth quantities of certain *Bacillus* sp. isolated from soil. *Turk. Elec. J. Biotech.,* 36: 24-30.

Bora L. 2012. Polyhydroxybutyrate production accumulation in *Bacillus megaterium* and optimization of process parameters using response surface methodology. *J. Polym. Environ.,* 21: 415-420.

Contreras, A.R.I., M. Koller, M.M.S. Dias, M.C. Monfort, G. Braunegg and M.S.C. Calvo. 2013. High production of poly (3 hydroxybutyrate) from a wild *Bacillus megaterium* Bolivian strain. *J. Appl. Microbiol.,* 114: 1378-1387.

Doetsch, R.N. 1981. Determinative methods of light microscopy. In: *Manual of Methods for General Bacteriology*. (Eds.): Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg and G.B. Phillips. American Society of Microbiology, Washington, D.C. pp. 22-31.

Eversloh, T.L., K. Bergander, H. Luftmann and A. Steinbuchel. 2001. Identification of new class of biopolymer: bacterial synthesis of a sulfur-containing polymer with thioester linkages. *Microbiology,* 147: 11-19.

Flora, G.D., K. Bhatt and U. Tuteja. 2010. Optimization of culture conditions for poly- hydroxybutyrate production from isolated *Bacillus* species. *J. Cell Tissue. Res.,* 10: 2235-2242.

Gouda, K.M., E. Swellam, F. Azza and H.S. Omar. 2011. Production of PHB by *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen source. *Microbiol. Res.,* 156: 201-207.

Grothe, E., M.M. Young and Y. Chisti. 1999. Fermentation optimization for the production of poly (hydroxy butyric acid) microbial thermoplastic. *Enz. Microbiol. Technol.,* 25: 132-141.

Halami, P.M. 2008. Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06. *World J. Microbiol Biotechnol*., 24: 805-812.

Hashimoto, K., H. Tsuboi, S. Iwasaki and Shirai. 1993. Effect of pH on the production of poly (β) hydroxybutyrate by photosynthetic bacterium, *Rhodospirillu mrubrum*. *J. Chem. Eng. Tech.,* 3: 227-232.

Kemp, W. 1989. Organic Spectroscopy. 3rd ed. Macmillan Education Ltd., Hong Kong.

Khare, A. and S. Deshmukh. 2006. Studies toward producing eco-friendly plastics. *J. Polm Film. Sheeting,* 22: 193-211.

Kominek, L.A. and H.O. Halvorson. 1965. Metabolism of poly-β-hydroxybutyrate and acetoin in *Bacillus cereus. J. Bacteriol.,* 90: 1251-1259.

Kulpreecha, S., A. Boonruangthavorn, B. Meksiriporn and N. Thongchu. 2009. Inexpensive fed-batch cultivation for high poly (3-hydroxybutyrate) production by a new isolate of *Bacillus megaterium. J. Biosci. Bioeng.,* 107: 240-245.

Kumaravel, S., R. Hema and R. Lakshmi. 2010. Production of Polyhydroxybutyrate (Bioplastic) and its Biodegradation by products. *Global Adv. Res. J. Microbiol.,* 2: 54-064.

Law, J.H. and R.A. Slepecky. 1992. Assay of polyhudroxybutyric acid. *J. Bacteriol.,* 82: 33-36.

Liu, M., J.E. Gonzalez., L.B. Willis and G.C. Walker. 1998. A novel screening method for isolating exopolysaccharides deficient mutants. *Appl. Environ. Microbial.,* 64: 4600-4602.

Lopez, A.J., M.J. Naranjo, J.C. Higuita, A.M. Cubitto, A.C. Cardona and A.M. Villar. 2012. Biosynthesis of PHB from a new isolated *Bacillus megaterium* strain: outlook on future developments with endospore forming bacteria. *Biotechnol. Bioprocess. Eng.,* 17: 250-258.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.,* 31: 426-28.

Nakata, H.M. 1963. Effect of pH on intermediates produced during growth and sporulation of *Bacillus cereus. J. Bacteriol.,* 86: 577-581.

Patel, S.K., M. Singh and V.P. Kalia. 2011. Hydrogen and Polyhydroxybutyrate producing abilities of *Bacillus* spp. from glucose in two stage system. *Indian J. Microbiol.,* 51: 418-423.

Ramadas, N.V., R. Sindhu, P. Binod and A. Pandey. 2012. Development of a novel solid-state fermentation strategy for the production of poly-3-hydroxybutyrate using polyurethane foams by *Bacillus sphaericus* NII 0838. *Ann. Microbiol.,* 63: 1265-1274.

Senthil, K. B. and G. Prabakaran. 2006. Production of PHB (bioplastics) using bio-effluent as substrate by *Alcaligens eutrophus*. *Ind. J. Biotechnol.,* 5: 76-79.

Singh, G., A. Kumari, A. Mittal, V. Goel, A. Yadav and N.K. Kumar. 2012. Cost effective production of Poly-β-hydroxybutyrate by *Bacillus subtilis* NG05 using sugar industry waste water. *J. Polym. Environ.,* 21: 441-449.

Snedecor, G.W. and W.G. Cochran. 1989. Statistical method. 7th ed. Low State University.

Steinbuchel, A., E. Hustede, M. Liebergesell, U. Pieper, A. Timm and H. Valentin. 1992. Molecular basis for biosynthesis of polyhydroxyalkanoaic acids in bacteria FEMS. *Microbiol. Rev.,* 103: 217-230.

Thirumala, M., S.V. Reddy and S.K. Mahmood. 2010. Production and characterization of PHB from two novel strains of *Bacillus* spp. isolated from soil and activated sludge. *Indian J. Microbiol., Biotechnol.,* 37: 271-278.

Tsuge, T., K. Tanaka, M. Shimoda and A. Ishizaki. 1999. Optimization of lactic acid feeding for the production of Poly-D-3-hydroxybutyric acid by *Alcaligenes eutrophusin* fed batch culture. *J. Biosci. Bioeng.,* 88: 404-409.

Valappil, S.P., A.R. Boccaccini, C. Bucke and I. Roy. 2007a. Polyhydroxyalkanoates in Gram-positive bacteria: insight from the genera *Bacillus* and *Streptomyce*s. *Antonie van Leeuwenhoek,* 91: 1-17.

Valappil, S.P., K.S. Misra, R.A. Boccaccini, T., Keshavarz, C. Bucke and I. Roy. 2007b. Large scale production and efficient recovery of PHB with desirable properties, from newly characterized *Bacillus cereus* SPV. *J. Biotechnol*., 132: 251-258.

(Received for publication 27 February 2015)