JEB
Journal of Environmental Biology

Journal Website : www.jeb.co.in
E-mail : editor@jeb.co.in
ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Cost-effective substrates for production of poly-β-hydroxybutyrate by a newly isolated *Bacillus cereus* PS-10

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Publication Info

Paper received:
26 April 2014

Revised received:
20 September 2014

Re-revised received:
14 November 2014

Accepted:
25 November 2014

Abstract

Poly-β-hydroxybutyrate (PHB) may serve as one of the imperative substitutes for petroleum derived plastics because of their close functional analogy and biodegradation quality. In the present study, PHB producing ability of bacterial isolates was examined on low-cost agro industrial residues. Isolate PS-10 from domestic waste landfills, identified as *Bacillus cereus* PS-10 produced and accumulated appreciable amount of PHB. *Bacillus cereus* PS-10 was capable of using a wide variety of agro-based residues viz. maize bran, rice husk, wood waste, molasses, whey etc. as cost-effective carbon sources for PHB production. Molasses at 3% (w/v) supported maximum PHB production (9.5 gl^{-1}) and was followed by glycerol (8.9 gl^{-1}) at 2% (w/v). Certain carbon sources like almond shell powder and walnut shell powder are being reported for the first time for PHB production and supported reasonable PHB yield i.e. 6.6 and 4.6 gl^{-1}, respectively. Different cost-effective nitrogen sources like corn steep liquor, chick pea bran, soy bean meal, mustard cake etc. were used for PHB production. Highest PHB production was observed at pH 7 (9.6 gl^{-1}) after 48 hrs of fermentation, although *B. cereus* PS-10 grew and produced PHB over pH range of 5-9. Optimum inoculum level for maximum PHB production was found to be 5% v/v (A_{600} 0.9; approximately 10^8 cfu ml^{-1}). Fourier transform infrared spectroscopy (FT-IR) analysis of the extracted PHB showed characteristic peaks (1721.95, 1632.19 and 2926.43 cm^{-1}) similar to standard PHB. Melting point of PHB was found to be 185°C. *Bacillus cereus* PS-10 may be a sound PHB producer, especially by exploiting low cost substrates.

Key words

Agroindustrial residues, *Bacillus cereus*, Cost-effective carbon sources, PHB production

Introduction

Polyhydroxyalkanoates (PHAs) represent natural, renewable, biocompatible and fully degradable biopolymers that consist of hydroxyalkanoic acid monomers. These are produced by wide range of bacteria as energy storage compounds, especially during limited nutritional supplies and in the presence of excess carbon source (Kulkarni *et al.,* 2011; Hungund *et al.,* 2013). PHAs due to their thermoplastic and elastomeric properties can be transformed into plastics like materials similar to petrochemical derived plastics, but fully biodegradable. Poly-β-hydroxybutyrate (PHB) represents one of the most studied PHA, and could be one of the strong contenders for bioplastic production. Applications spectrum of PHB have been extended to pharmaceutical products used in surgery, osteosynthetic materials, bone plates, surgical sutures, tissue engineering, pharmacology and many other materials in medicine (Shivakumar, 2012). PHB being insoluble in water, resistant to UV radiation and impermeable to oxygen, can be used as food packaging material (Chauhan *et al.,* 2013). Several bacterial species have been reported to accumulate PHB viz., *Halomonas campisalis* (Kulkarni *et al.,* 2011), *Haloferax mediterranei* (Bhattacharyya *et al.,* 2012), *Alcaligenes latus, Ralstonia eutropha, Azotobacter beijerinckii, Bacillus megaterium*, *Pseudomonas oleovorans etc.* (Bhuwal *et al.,* 2013). β-proteobacterium *Ralstonia eutropha* is known as the model organism for PHA production, as it can store high amount of PHA *i.e.,* up to 90% of its cell dry weight under nutrient limitation in the presence of ample carbon source as PHB (Riedel *et al.,* 2012). Some Gram-negative bacteria like *Alcaligenes*, *Ralstonia* and

Pseudomonas, Halobacterium, *Haloarcula* show reasonably good PHB accumulation (Singh *et al.,* 2013) but Gram-positive bacteria may be preferred when the intended application of PHB is in biomedicine.

PHB production from Gram-negative bacteria may necessitate extra purification steps for eliminating potential contamination by endotoxins, while no such purification is required when producing PHB from Gram-positive bacteria as these are devoid of outer membrane of lipopolysaccharide (Lopez *et al.,* 2012). Among several Gram-positive bacteria *viz. Clostridium*, *Corynebacterium*, *Nocardia*, *Bacillus*, *Rhodococcus*, *Streptomyces* and *Staphylococcus* reported for PHB production, *Bacillus* spp. offer several advantages and have been explored for wide range of industrial products including PHB (Singh *et al.,* 2013). *Bacillus* spp. have relatively fast growth and capability to utilize wide range of agro-industrial wastes as substrates (Masood *et al.,* 2012) due to their ability to secrete variety of extracellular enzymes such as cellulases, xylanases, amylases and proteases (Bajaj and Singh, 2010). Furthermore, *Bacillus* spp. represents a model system for heterologus expression of foreign genes associated with PHA production and several fine chemicals (Law *et al.,* 2003; Schallmey *et al.,* 2004). However, PHB production from *Bacillus* strains may be affected due to induction of sporulation under nutrient limited fermentation conditions (Lopez *et al.,* 2012) and economic constrains involving soaring production costs (Valappil *et al.,* 2007).

In the present study, a range of economic carbon and nitrogen sources were examined for PHB production from a newly isolated proficient PHB producing bacterium *Bacillus cereus* PS-10. PHB produced was analysed by FTIR.

Materials and Methods

Isolation and screening of PHB producing bacteria: Samples from diverse sources *viz.*, domestic waste landfills, soil of paddy fields, root nodules of leguminous plants, hot spring soil, compost, cafeteria waste, tea field soil, automobile washing area soil, garden soil *etc.,* were collected from different locations of Jammu and Kashmir and Himachal Pradesh during May-December 2011. Isolation of PHB producing bacteria was accomplished based on the methods of Khanafari *et al.* (2006) with slight modifications. Briefly, samples were inoculated @ 5% w/v nutrient broth and enriched while shaking at 30 ºC for 24 hr in an incubator shaker (Innova, New Brunswick, USA). The enriched broth was appropriately diluted and spread plated on whey agar containing (gl^{-1}): 0.1 $MgSO_4.7H_2O$; 0.5 K_2HPO_4; 0.1 NH_4NO_2; 20.0 glucose; 0.5 malt extract; 1.0 yeast extract; 20.0 agar; (pH 7), 1 l whey and 0.5 µg ml^{-1} Nile blue A. Plates were incubated at 30°C for 2-3 days and colonies appeared were exposed to UV light and those showing orange fluorescence under UV were earmarked as presumptive PHB producers.

PHB producing ability of bacterial isolates was examined further by confocal microscopic analysis by using Nile red, a lipophilic dye (Model Olympus Fluoview Ver 1-7b) as described by Degelau *et al*. (1995). The cells were first grown in PHB production medium (PPM) which consisted of (gl^{-1}): 0.1 $MgSO_4.7H_2O$; 0.5 K_2HPO_4; 0.1 NH_4NO_2; 20.0 glucose; 0.5 malt extract; 1.0 yeast extract; 7 pH for 48 hr. Cells were harvested and immediately stained with 0.1–0.5 volumes of Nile red solution (0.1–1 mg ml^{-1} in ethanol. Nile red was excited with 543 nm green helium-neon laser and 488 nm argon laser. PHB granules were observed in cells under the phase-contrast illumination mode at 60X magnification and all the digital images were captured.

Fermentation for PHB production : All the PHB producing isolates were subjected to submerged fermentation in PPM for PHB production. Inoculum was developed by cultivating the cells in PPM under shaking at 30°C for 18 hr to attain absorbance (A_{600}) of 0.8-0.9 (approx. 10^8 cfu ml^{-1}) and inoculated @ 5% (v/v) into PPM for PHB production. Fermentation was executed at 30°C under shaking at 150 rpm. Fermented broth was centrifuged (Eppendorf centrifuge 5804-R, Germany) at different time intervals (24-96 hr) and the resultant biomass was used for PHB extraction.

Extraction and quantification of PHB : PHB extraction from bacterial cells was done with sodium hypochlorite digestion method (Demain and Davis, 1999). Biomass was suspended in sodium hypochlorite solution for 1 hr at 37ºC for complete digestion of cell components except PHB. Samples were centrifuged at 8,000 g for 20 min to collect PHB granules. Precipitated PHB was washed with acetone, water and dissolved in chloroform. Then it was allowed to evaporate overnight and dried pellet obtained was used for PHB estimation. Finally, granules were mixed with 5 ml of concentrated H_2SO_4 and the tubes were capped and heated for 10 min at 100ºC in a water bath to convert PHB to crotonic acid. Absorbance of the solution was read at 235 nm against sulphuric acid blank. PHB concentration was determined from an established standard curve and PHB production was estimated spectrophometrically (UV1800, Shimadzu, Japan) at 235 nm (Law and Slepecky, 1961).

Identification of selected isolate : Among the bacterial isolates screened for PHB production, the one which generated maximum PHB was selected and examined based on morphological, physiological and biochemical properties. Biochemical analysis included carbohydrate fermentation tests, Indole, methyl red and Voges-Proskauer test (IMVIC), catalase, amylolytic and proteolytic activity tests. Furthermore, the identity of bacterial isolate was confirmed based on 16S rDNA sequence analysis. The universal primers (16sF-5' AGA GTT TGA TCC TGG CTC AG 3' and 16s R-5' TGC GGY TAC CTT GTT ACG ACT 3') used for PCR amplification of 16S rDNA and reaction conditions employed were: 95 ºC for 4 min, 92ºC for 1 min, 56.4 ºC for 30 sec, 72 ºC for 1min, and 72 ºC for 7min. PCR product was eluted with Axygen

DNA gel elution kit and the purified product was sequenced with Applied Biosystems Instrument (Centre for Instrumentation Facility, University of Delhi, South Campus, New Delhi). The sequence was subjected to Blast analysis (NCBI) and aligned using ClustalW and dendrogram was generated (Mega 5).

PHB production by using various carbon and nitrogen sources : For determining the effect of carbon source on PHB production, glucose of PPM was replaced with either of the 26 carbon sources viz., rice husk, wheat bran, molasses, malt, whey, wood waste, sesame oil cake, cotton cake, potato peel powder, walnut shell powder, malt spent wash, almond shell powder, wheat waste, maize bran, glycerol, lauric acid, citric acid, acetone, castor oil, starch, ortho-phosphoric acid, mannitol and butyric acid at 20 gl^{-1} (w/v), and fermentation was carried out. Similarly, yeast extract of the initial PPM was replaced with various nitrogen sources like corn steep liquor, mustard cake, peptone, chick pea bran, soy bean meal, casein, urea, gelatine, and $(NH_4)_2SO_4$ (@ 1 gl^{-1}) to determine the effect of nitrogen source on PHB production. Fermentation was executed and samples withdrawn at different time periods were analysed for PHB (Law and Slepecky, 1961). To find out the optimum level of best carbon source (molasses, glycerol) its concentration was varied in PPM (5 to 50 gl^{-1}) and PHB yield was analyzed after fermentation. Similarly, best selected nitrogen source (peptone) was used at different concentrations in PPM (0.5 to 5 gl^{-1}) and PHB production was determined. Total carbohydrate content of molasses was estimated by phenol sulphuric acid method (Dubois *et al.,* 1956).

Effect of initial medium pH : To examine the effect of initial medium pH on PHB production, PPM containing molasses as carbon source (30 gl^{-1}) and peptone as nitrogen source (1 gl^{-1}) was adjusted at pH 5 to 9 with 2 N HCl or NaOH. Freshly grown *B. cereus* PS10 was inoculated into PPM and fermentation was conducted up to 96 hr and PHB was extracted and estimated.

Effect of inoculum size on PHB production : *B. cereus* PS10 was cultivated in PPM at 30 ºC and 150 rpm to attain biomass level of approximately 10^8 cfu ml^{-1} (A_{600} 0.9). Biomass was inoculated in PPM at different levels (2-10 %, v/v) and fermentation was carried out under usual conditions and PHB yield was monitored for 24-96 hr.

Fourier transform-infrared spectroscopy (FT-IR) and polymer melting point analysis : PHB production from *B. cereus* PS-10 was executed under submerged fermentation and PHB produced was subjected for IR spectra recording in the range 4000-400 cm^{-1} at a resolution of 4 cm^{-1} to confirm the functional groups of the extracted polymer as per the method described by Nichols *et al*. (1985). Melting point of polymer was estimated to examine polymer stability at higher temperature (BUCHI, Melting Point B-545, Switzerland). All the experiments were performed in triplicates and all the data were represented as Mean ± SD.

Results and Discussion

PHB producing bacterial colonies showed bright orange fluorescence under UV light on Nile Blue A containing medium. Confocal microscopic analysis of PHB producing bacterial cells indicated the appearance of cells filled with red color PHB granules. Alternate staining methods like direct staining of colonies with sudan black have been used by some researchers for detection of PHB granules (Singh *et al.,* 2013).

After initial screening, all the 31 isolates were subjected to submerged fermentation in PHB production medium. Bacterial isolate PS-10, isolated from domestic-waste landfills was standalone, showed substantial PHB production (8.6 gl^{-1} ±0.25) after 48 hr of fermentation as examined by spectrophotometric analysis while all other bacterial isolates yielded PHB < 3.5 gl^{-1}. Therefore, bacterial isolate PS-10 was selected for further studies including its identification by morphological and biochemical means. Macroscopic analysis showed that bacterial colonies were sticky, discrete and creamy white. Microscopic examination established that bacteria were Gram-positive, rod shaped and sporulating. The isolate was identified using a series of biological tests. Carbohydrate utilization test for 36 different sugars showed that the bacterial isolate PS-10 effectively utilized maltose, fructose, dextrose, glycerol, salicin, rhamnose and glucose. Besides, isolate PS-10 was catalase and methyl red positive but negative for VP test, hydrolysed casein, starch and lipids. On the basis of these characteristics, PS-10 was identified as *Bacillus* sp. and designated as *Bacillus* PS-10. Furthermore, 16S rRNA gene sequence of *Bacillus* PS-10 showed maximum homology (99%) with that of several *Bacillus cereus* strains available in NCBI GenBank data base indicating that the isolate *Bacillus* PS-10 was most probably a strain of *B. cereus* (Fig. 1). Phylogenetic analysis was carried out and sequence was deposited at GenBank (NCBI, Bethesda, MD, USA; *http://blast.ncbi.nlm.nih.gov/Blast.cgi*) under the accession no. KF499032.1.

PHB from Gram-positive bacteria may be promising for biomedical and tissue engineering applications (Lopez *et al.,* 2012) as Gram-positive bacteria are devoid of lipopolysaccharides and there is no chance of lipopolysaccharides contamination. Time-course (24-96 hrs) analysis for PHB production indicated that PHB was a growth-associated product and its accumulation significantly increased during the exponential phase and reached maximum after 48 hr (8.6 ±0.25 gl^{-1}). Maximum PHB yield was achieved after 48-72 hr of fermentation. After 72 hr, a decrease in the level of cell dry weight coincided with a small decrease in PHB content. During adverse conditions PHB is used by cell as an internal reserve of carbon and energy. Furthermore, increase in viscosity of the medium may also lead to unfavourable conditions such as production of extracellular metabolites, presence of an intracellular PHB depolymerase, depletion of essential nutrients in the medium or self utilization of PHB by bacteria due to nutrient depletion and cells consumption of PHB as a carbon source

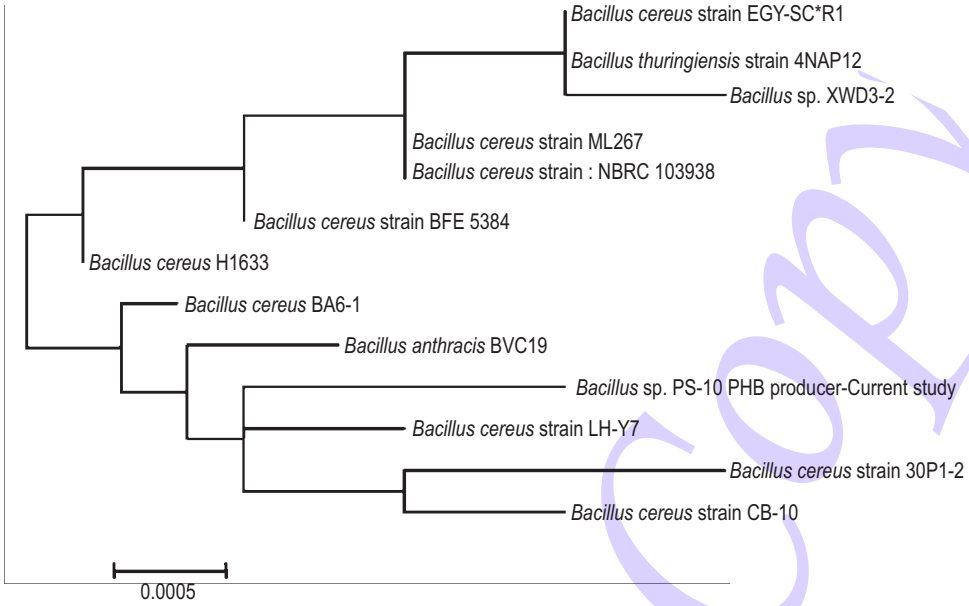

Fig. 1 : Phylogenetic tree showing comparison of 16S rDNA sequence of bacterial isolate PS-10 with that of other spp. of *Bacillus* in NCBI database

(Madison and Huisman, 1999; Prasanna *et al.,* 2011). Fermentation time for maximum PHB production varies among different bacteria and depends largely upon cultural/ environmental conditions employed during fermentation and genetic make-up of the organism.

Bacillus cereus PS-10 seems to have versatile metabolic potential as it utilized a wide range of carbon sources for growth and PHB production. Among various carbon sources, glycerol and molasses supported highest PHB production *i.e.,* 8.9 and 8.6 gl^{-1} after 72 and 48 hr, respectively. However, substantial PHB yield (3-6.6 gl^{-1}) was obtained on other carbon sources like acetone, ethanol, starch, rice husk, potato peels powder, wheat bran, sesame cake, walnut shell powder, maize bran, whey and almond shell powder. Carbon sources like walnut shell powder and almond shell powder are being reported for the time, and showed promising results for PHB production by *B. cereus* PS10. But some carbon sources like wood waste, citric acid, butyric acid, sawdust gave comparably low yield (0.8-1.3 gl^{-1} ±0.02-0.28). Nonetheless, it was established that organism *B. cereus* PS-10 has immense enzymatic/metabolic potential by virtue of which it was able to degrade complex carbon sources for growth and PHB production. Time-profile (24-96 hr) for PHB production on glycerol was (gl^{-1}): 5.2, 8.3, 8.9 and 6.4; while on molasses PHB yield was 3, 8.6, 5.2 and 3.3 after every 24 hr interval.

PHB synthesis and utilization are closely connected with energy requirements of cell including the nature of carbon utilized. It is desirable that PHB production be attempted by using inexpensive agricultural or other residues as carbon source to substantially reduce the cost of PHB production, considering carbon source as a major cost determining factor for PHB production (Valappil *et al.,* 2007). PHB production has been reported from several bacteria by using waste materials or agricultural residues as inexpensive carbon sources (Van-Thuoc *et al.,* 2008; Yezza *et al.,* 2006). Furthermore, readily available low-price carbon sources may have high amount of nutrients, such as amino acids and peptides, which contributes to improved cell growth and metabolite biosynthesis and pave the way for resourceful and cost-effective production of PHB. PHB being the reserved food polymer produced during the time of starvation is degraded to provide carbon and energy when external carbon source is exhausted (Lopez *et al.,* 2012). Sugar industry waste water with nutritive adjustment has been used for PHB production from *Bacillus subtilis* NG220 (Singh *et al.,* 2013). Agro-industrial residues like wheat bran, potato starch, sesame oil cake, groundnut oil cake, cassava powder, jackfruit seed powder and corn flour were examined for selecting the best substrate for PHB production by *Bacillus sphaericus* NCIM 5149 (Ramadas *et al.,* 2009). Similarly, different carbon sources like soya flour, CMC, bagasse, molasses, wheat bran , wheat germ, rice bran and ragi bran were used for PHB production from *Bacillus thuringiensis* IAM 12077 (Shivakumar, 2012). However, *B. megaterium* showed maximum PHB accumulation when glucose was used as carbon source (Hori *et al*., 2002). *Bacillus* species has been reported to accumulate PHB up to 90% of the cell dry weight by utilizing soy molasses oligosaccharides like raffinose without the need for nutrient limitation (Full *et al.,* 2006).

In another experiment, molasses and glycerol which supported maximum PHB production from *B. cereus* PS-10 were examined at different concentrations (5–50 gl^{-1}) to elucidate their optimum levels. PHB production increased with increase in the concentration of molasses and reached maximum (9.5 gl^{-1}) at 30 gl^{-1} of molasses after 48 hr of fermentation (Fig. 2a). However, substantial PHB yield (8.6- 9.1 gl^{-1}) could be seen at molasses concentration of 20-35 gl^{-1} but outside this range PHB yield was reduced. In addition, it was observed that the optimum fermentation time for maximum PHB production was 48 hr after prolonged incubation PHB yield decreased.

The system has substrate inhibition with higher molasses concentration showed adverse effect on production of PHB. Molasses, which is a common industrial by-product from sugar industries, is rich in nutrients and minerals and at the same time it is cheap and available in plenty and can be exploited for the production of a variety of industrial products (Bajaj and Sharma, 2010). However, molasses may have growth inhibitory compounds as well; *B. cereus* PS-10 is remarkable as it not only grow well in molasses but produced substantial PHB content. Molasses has been used as a carbon source for PHB production, and presence of growth factors like organic acids, vitamins and minerals in molasses may enhance PHB yield (Tripathi *et al.,* 2013). Similar to the current study, Gouda *et al.* (2001) used different sugarcane molasses levels (1–5%, w/v) for growth of *B. megaterium* and production of PHA/PHB after 48 hrs of incubation. The best growth was obtained with 3% molasses, while maximum yield of PHA and PHB was obtained with 2% molasses, and then decreased with increasing the level of molasses upto 5%. Chaijamrus and Udpuay (2008) found molasses as a favourable carbon source for PHB production and highest growth (7.2 gl^{-1}) was obtained at 4% (w/w) molasses.

Similarly, glycerol was used at varying concentration (5-50 gl^{-1}) for PHB production from *B.cereus* PS-10, and it was observed that glycerol at 20 gl^{-1} gave maximum amount of PHB *i.e.* 8.9 gl^{-1} after 72 hrs of fermentation (Fig. 2b). However, considerable PHB yield (7.8 gl^{-1} and 7.7-7.2 gl^{-1} was obtained at 15 gl^{-1} and 25-30 gl^{-1} of glycerol, respectively. However, higher level of glycerol (above 35 gl^{-1}) in the medium lead to yield reduction of PHB. But the capability of bacteria to grow at such higher concentration of glycerol indicates its osmotolerant nature.

Nowadays, glycerol being a principal by-product generated by growing biodiesel industries offers an inexpensive carbon source for production of value-added biodegradable PHB. Zhu *et al.* (2009) explained that by regulating glycerol content, different lengths of PHB can be produced to meet the diverse criteria of various industrial and medical applications. The concentration of glycerol needs to be strictly controlled while using glycerol as carbon source. Increasing glycerol concentration results in a gradual reduction of biomass, PHB yield and molecular mass of PHB. *Burkholderia cepacia* ATCC 17759 synthesized PHB from glycerol concentrations ranging from 3% to 9% (v/v). Sindhu *et al.* (2011) used nitrogen-limited medium with different crude glycerol as sole carbon source ranging from 1-5% (v/v) and found that maximum PHB yield was

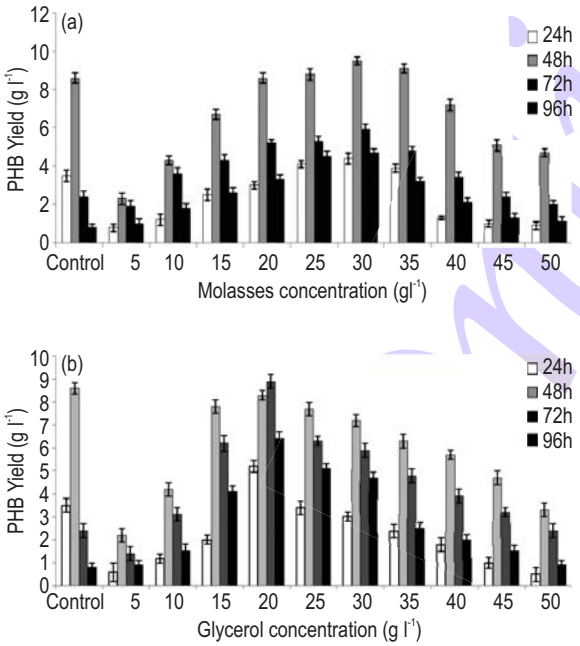

Fig. 2 : PHB production by *Bacillus cereus* PS-10 using various concentrations of (a) molasses (5-50 g l^{-1}) and (b) glycerol (5-50 g l^{-1})

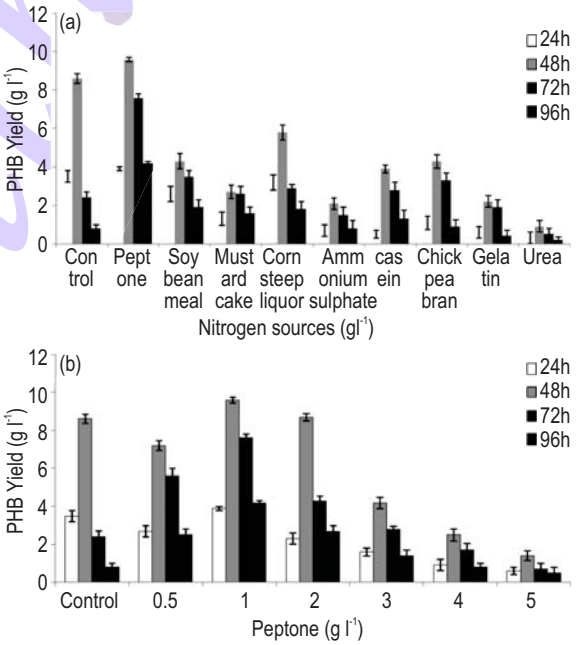

Fig. 3 : PHB production by *Bacillus cereus* PS-10 (a) using different nitrogen sources and (b) at different concentrations of peptone (0.5-5 g l^{-1})

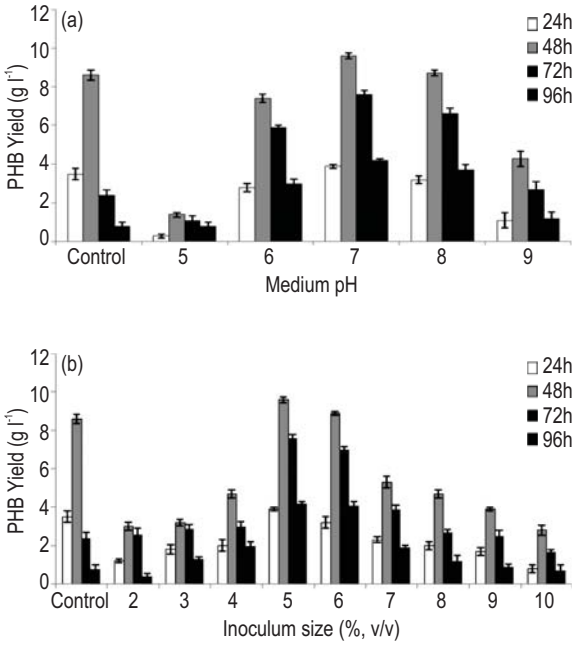

Fig. 4 : PHB production by *Bacillus cereus* PS-10 (a) using media at different pH (5-9) and (b) varying level of inoculum size

obtained at 1% glycerol concentration. This might be attributed to substrate inhibition at higher concentration.

Among various nitrogen sources examined, peptone supported maximum PHB production (9.6 gl^{-1}) *i.e.,* higher than control (8.6 gl^{-1}) after 48 hr of fermentation (Fig. 3a). Other crude and refined nitrogen sources supported growth of bacteria but nonetheless, PHB yield was much less than that of control. Corn steep liquor, chick pea bran, soy bean meal supported moderate PHB production, however, with casein and mustard cake low PHB yield was obtained. Urea and gelatin were relatively ineffective as nitrogen sources. Furthermore, peptone was examined at different concentrations (0.5-5 gl^{-1}) and it was observed (Fig. 3b) that 0.1% peptone was the optimum concentration for maximum PHB yield (9.6 gl^{-1}).

Nitrogen source is essential to meet growth requirement of cell (Bajaj and Abbass, 2011). Under normal conditions, bacteria synthesize their cell materials like proteins and grow. But in nutrient limiting conditions bacteria may shift their protein synthesis to PHB synthesis for survival. Nutrient limitation/ environmental stress are necessary to trigger PHB accumulation. PHB production needs excess carbon source in addition to nitrogen limitation (Elsayed *et al.,* 2013). Peptone was also found to be the most favourable nitrogen source for PHB production for *Bacillus* sp. (Singh *et al.,* 2011). In contrast to the present results, ammonium sulphate was reported to be the best nitrogen source for PHB production from different microorganisms such as *Alcaligenes eutrophus*, *Methylobacterium* sp. and *Sinorhizobium fredii*. Highest PHB was obtained from ammonium sulphate by halotolerant photosynthetic bacteria *Rhodobacter sphaeroides,* when cultivated under aerobic and dark conditions (Sangkharak and Prasertsan, 2008). Corn steep liquor was found to be the best nitrogen source for PHB synthesis by *Bacilus megaterium* (Gouda *et al.,* 2001).

Production of PHB in media at different pH showed that pH 7 favoured maximum PHB production (9.6 gl^{-1}), however, substantial amount of PHB yield was obtained at pH 8 and 6 (8.7 and 7.4 gl^{-1}) respectively (Fig. 4a), but amount of PHB yield was significantly reduced at pH 9.

The most favorable pH for bacterial growth is neutral pH (7.0), and hence contributes to higher PHB production (Shaaban *et al*., 2012). *Bacillus subtilis* NG220, an isolate from sugar industry waste water showed pH 7 as suitable pH for PHB production. Lack of polymer accumulation at higher pH value can best be explained by an effect on the degenerative enzymes of polymer breakdown, so that the PHB is utilized at the rate almost equal to the rate of its synthesis (Singh *et al.,* 2013). *Bacillus sphaericus* NCIM 5149 showed pH 7.5 as most appropriate for PHB production (Ramadas *et al.,* 2009), while for *Bacillus* species pH 6-7 was most effective for PHB accumulation (Singh *et al.,* 2011). However, *Alcaligenes eutrophus* had optimum pH between 6.8 and 8.0 for PHB production (Shivakumar, 2012).

Amount of initial biomass may influence the final yield of PHB. Current study showed that inoculum at 5%, (v/v) was best for maximum PHB yield (9.6 gl^{-1}). Higher or lower inoculum level resulted in yield reduction of PHB from *B. cereus* PS-10 (Fig. 4b).

Inoculum level is one of the most important fermentation parameters in production optimization studies. Low inoculum size required longer time for cells to multiply and produce the desired product. A small amount of inoculum can lead to insufficient number of microbial cells and reduced amount of PHB while a much higher inoculum can cause oxygen limitation and depletion of nutrients in the culture media (Abusham *et al.,* 2009). *Bacillus sphaericus* NCIM 5149 produced maximum PHB when inoculum was used at 2% (Ramadas *et al.,* 2009), while *Bacillus subtilis* NG220 did so at 1% v/v inoculum (Singh *et al.,* 2013). IR spectrum of the extracted polymer, illustrated bands at 1721.95 and 2926.43 cm^{-1} corresponding to aliphatic ester carbonyl C=O of RCOA and C-H stretch, respectively. Other absorption bands at 3433.35 and 2376.02 cm^{-1}, showed the presence of O-H bonding and C-H bond of aliphatic compounds. Band found at 1721.95 and 1632.19 cm^{-1} corresponded to ester carbonyl group and to –CH group. Band at 1721.95 cm^{-1} was considered as diagnostic signal for PHB. Band found at 1,380.17 cm^{-1} was assigned to symmetric wagging of CH_3 groups. Band at 1,156.33 cm^{-1} was characteristic of the symmetric stretching vibration of C–O–C group.

The remaining bands located at 1000 ~ 1300 cm^{-1} correspond to stretching of C–O bond of ester group. The results described above are congruent with the findings reported previously. The most prominent marker (ester carbonyl) band for PHB was at 1740 cm^{-1} and 1724.03 cm^{-1} for pure PHB (Lopez-Cortes *et al.,* 2010). The region of 1675–1735 cm^{-1} was associated with C-O stretching of ester carbonyl bond. FTIR spectra of PHA produced by *Bacillus circulans* (MTCC 8167) showed high absorbance at 3360, 2922, 1735 and 1206 cm^{-1}, which resulted in the vibration functions of OH, CH, CO and COC, respectively (Zribi-Maaloul *et al.,* 2013).

In pure PHB granule, asymmetrical deformation of C-H bond in CH_2 groups and CH_3 groups C=O bond stretching and C-O ester bond were represented by wave numbers 1460, 1379, 1726 and 1150 cm^{-1}, respectively. Sharp peaks at wave number 1727, 1455 and 1381 cm^{-1} which corresponds to C=O bond stretching, C-O ester bond and asymmetrical deformation of C-H bond in CH_2 and CH_3 groups of PHB were similar to IR spectra of pure PHB (Tripathi *et al.,* 2013).

Melting point of extracted PHB was tested and it was observed that the polymer started melting at 181.3°C and melted completely at 185°C. This showed that the polymer had good stability at high temperature and this theromostability is desired for potential applications for production of bioplastic.

The present study concludes that *Bacillus cereus* PS-10 has enormous enzymatic/metabolic potential to utilize variety of cost-effective carbon sources for growth and PHB production. Furthermore, PHB produced possessed remarkable characteristics like thermostability and high melting point from industrial application view-point.

Acknowledgments

Financial support from the Council of Scientific and Industrial Research (CSIR), Govt of India, in the form of GRANT-IN-AID RESEARCH PROJECT (Ref: No.37(1378)/09/EMR-II, dated 12-10-09), to Dr. Bijender K. Bajaj and senior research fellowship to Ms. P. Sharma is gratefully acknowledged. Authors thank Director, School of Biotechnology, University of Jammu, Jammu for laboratory facilities and SAIF facility, Punjab University Chandigarh and Indian Institute of Integrative medicine (CSIR), Jammu for facilitating some analytical work.

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