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Analysis and Evaluation of Growth Parameters for Optimum Production of Polyhydroxybutyrate (PHB) by *Bacillus thuringiensis* strain CMBL-BT-6

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Abstract.- Synthetic polymers obtained from petrol causes air pollution only because they are not degraded in soil for a long time. For this reason, a microbial plastic poly-β-hydroxybutyrate (PHB) has gained importance since it can be easily degraded in nature. PHB is a widely distributed intracellular reserve substance typical of prokaryotes. PHB exists in the cytoplasmic fluid in the form of crystalline granules about 0.5 µm in diameter and can be isolated as native granules or by solvent extraction. The study aimed at screening of PHB producing strain and optimizing media parameters for increased PHB production by a *Bacillus thuringiensis* strain CMBL-BT-6, which was identified as PHB producing strain by staining with Sudan Black B dye. Conditions for best PHB production were optimized. Maltose was found to be best carbon source and ammonium nitrate the best nitrogen source producing 0.867g/L and 0.953g/L of PHB, respectively. Glucose was used as a substrate and 4% glucose concentration (0.953g/L) was found to be best for PHB production. The data further indicated that CMBL-BT-6 produced 2.75g/L PHB at pH 7.0, when incubated at 37°C for 24 h.

Keywords: Bioplymers, poly-β-hydroxybutyrate, *Bacillus thuringiensis* strain, crotonic acid, high performance liquid chromatography.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a class of natural polyesters, which can be produced and accumulated by many Gram-positive and Gram-negative bacteria from at least 75 different genera. These polymers are accumulated intracellularly under conditions of nutrient stress and act as a carbon and energy reserve (Steinbüchel, 2001; Reddy *et al*., 2003). Polyhydroxybutyrate (PHB) is commonly studied and best characterized PHA. It is accumulated when carbon and energy sources are in excess, but growth is limited by the lack of oxygen, nitrogen, or phosphorus source. The characteristic of this compound is similar to synthetic plastic or petrochemical-based plastics such as polypropylene, polyurethane, vinyl chloride and hexachloroethane etc. PHB and its copolymer can be used as biodegradable plastic, which can reduce the current problems with decreasing fossil resources and environmental impact caused by plastic garbage (Luengo *et al*., 2003). In addition, it has a promising application in agriculture, medicine and material science (He *et al*., 2004). However, the important factor preventing the industrial and commercial production of PHB is its high price of production compared to synthetic plastic. Improved cultivation medium and conditions are, therefore, required for reducing the cost (Khanna and Srivastava, 2005a). The PHB production capacities of bacteria have been investigated for possible application in industry (Lee, 1996; Hanzlikova *et al*., 1985; Nickerson *et al*., 1981; Lach *et al*., 1990).

Halotolerant bacteria have been reported to produce high amounts of PHAs 40-60% dry cell weight (DCW) were accumulated in halotolerant bacteria under the starvation condition (Khatipov *et al*., 1998; Luengo *et al*., 2003; Hassan *et al*., 2004; Chen *et al*., 2006). Recently, the highest PHA production was obtained from *Ralstonia sphaeroides* strain 14F which showed 3.5 g/l PHA,

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60% DCW cultivated in modified growth medium where malate was substituted by 5 g/L fructose under two stage aerobic dark condition (Lorrungruang *et al*., 2006).The bacterium capable of producing PHB has been identified in more than 20 bacterial genera, including *Azatobacter, Bacillus, Beijernickia, Alcaligenes, Pseudomonas, Rhizobium* and *Rhodospirillum*. Many researchers have explained that soil bacteria generally produce PHB (Lee, 1996; Hanzlikova *et al*., 1985; Nickerson *et al*., 1981; Lach *et al*., 1990). Reusch and Sadoff (1983) have shown that PHB is an important molecule in cytoplasm and in cell walls. *Bacillus* species have been shown to accumulate PHB during the sporulation of bacterial growth.

Because of the ubiquitous use of petroleum-based plastics, and damaging effect of their persistence in the environment and their fossil-fuel derivatives, alternate to these traditional plastics are being explored. Biodegradeable plastic is a promising alternative, which ends up into water and carbon dioxide in the environment after degradation. The present study focuses on determining optimum conditions for production of poly-β-hydroxybutyrate (PHB) granules by *Bacillus thuringiensis* strain CMBL-BT-6.

MATERIALS AND METHODS

Organisms

Bacillus thuringiensis CMBL-BT-6 was obtained from School of Biological Sciences (SBS), University of the Punjab, Lahore. The PHB producing capability of the organism was confirmed by Sudan black staining method (Burdon *et al*., 1942).

Nutrient agar medium (peptone 5 g/L, meat extract 1 g/L, yeast extract 2 g/L, sodium chloride 5 g/L, agar 15 g/L, pH 7.0) was used for strain cultivation. Nutrient broth used for PHB production had the same composition as that of nutrient agar medium except Agar.

Screening of PHB producing bacteria

Burdon *et al.* (1942) was followed with modifications for production of PHB by the BT strain. Smear of PHB producing bacteria was heat fixed on sterilized glass slide and stained with Sudan black B solution (0.3% solution (w/v) in 60% ethanol) for 10 min, rinsed with water and then counter-stained with 0.5% safranine for 5 seconds. Stained sample was observed under oil immersion at 100x magnification. The bacterial cells having dark blue color granules were taken as positive for PHB production.

Determination of dry cell mass (DCM)

The bacterial isolates from soil were grown in nutrient broth under the tested conditions. After 24 h of incubation at 37°C, 10 ml of culture was centrifuged at 4,185x g, pellet was washed first with phosphate buffer and then twice with sterile deionised water. The pellet was dried at 100°C for 24h. The dried material was incubated at 60°C for 1h with 5% (v/v) sodium hypochlorite and centrifuged at 4,185xg at 28-31 °C for 15 min to get DCM.

Quantification of PHB production

PHB was extracted from DCM using acetone-alcohol method and quantified according to Law and Slepecky (1961). The cell pellet containing PHB was again washed with acetone and ethanol. The polymer granules were dissolved in hot chloroform. The chloroform was filtered and to the filtrate, 10 ml hot concentrated H_2SO_4 was added. The addition of sulfuric acid converts the polymer into crotonic acid. The solution was cooled and the absorbance was taken at 235 nm against sulfuric acid as blank. By referring to the standard curve, the quantity of PHB produced was determined.

Optimization of culture conditions for maximum PHB production

Different growth conditions such as time, temperature, pH, carbon source, nitrogen source and substrate concentration affecting the PHB production were optimized.

Effect of temperature

Nine 250 ml conical flasks containing 100 ml nutrient broth containing 2% glucose and 0.1% ammonium nitrate, pH 7 inoculated with BT strain CMBL-BT-6 were incubated at different temperatures *viz.*, 4, 18, 21, 25, 28, 30, 35, 37 and 40°C for 24 h. The PHB produced in different flasks

was quantified spectrophotometrically as stated above. Based on the PHB yields, the best incubation temperature was selected. All optimization experiments were replicated three times and the values are indicated as mean ± SD in results section.

Effect of pH

Nine 250 ml conical flasks containing 100 ml nutrient broth containing 2% glucose and 0.1% ammonium nitrate at different pH 2, 3, 4, 5, 6, 7, 8, 9, and 10, inoculated with CMBL-BT-6 were incubated at 37°C for 24 h. The PHB produced in different flasks was quantified spectrophotometrically according to Law and Slepecky (1961) as stated above. Based on the PHB yields, the best pH was selected.

Effect of incubation time

Six 250 ml conical flasks containing 100 ml nutrient broth (pH 7.0) containing 2% glucose and 0.1% ammonium nitrate, inoculated with BT strain CMBL-BT-6 were incubated at 37°C for different time periods *viz.*, 24, 48, 72, 96, 120 and 144 h. The PHB produced in different flasks was quantified spectrophotometrically as described previously. Based on the PHB yields, the best time of incubation was selected.

Effect of different carbon sources

Seven 250 ml conical flasks containing 100 ml nutrient broth (pH 7.0) containing 2% glucose and 0.1% ammonium nitrate, each flask supplemented with one of different carbon sources such as glucose, fructose, sucrose, maltose, arabinose, xylose and lactose at a concentration of 1%, inoculated with BT strain CMBL-BT-6 were incubated at 37°C for 24 h. The PHB produced in each flask was quantified spectrophotometrically as described elsewhere. Based on the PHB yields, the best supplement carbon source was selected. The experiment was replicated three times.

Effect of different nitrogen sources

Seven 250 ml conical flasks containing 100 ml nutrient broth (pH 7.0) containing 2% glucose, 1% maltose and 0.1% ammonium nitrate, each flask supplemented with one of different nitrogen sources such as ammonium nitrate, ammonium citrate, ammonium sulphate, glycine, tryptone, urea and ammonium di-hydrogen phosphate at 0.1g/100 ml concentration, inoculated with BT strain CMBL-BT-6 were incubated at 37°C for 24 h. The PHB produced in each flask was quantified spectrophotometrically. Based on the PHB yields, the best supplement nitrogen source was selected..

Effect of different substrate concentrations

Seven 250 ml conical flasks containing 100 ml nutrient broth (pH 7.0) containing 1% maltose and 0.1% ammonium nitrate, each flask supplemented with one of the substrate (Glucose) concentrations *viz.*, 1, 1.5. 2. 2.5. 3, 3.5 and 4, inoculated with BT strain CMBL-BT-6 were incubated at 37°C for 24 h. The PHB produced in each flask was quantified spectrophotometrically according to Law and Slepecky (1961) as stated above. Based on the PHB yields, the best substrate concentration (3%) was selected.

HPLC analysis

About 500mg PHB produced by CMBL-BT-6 was digested in 1 ml of concentrated sulfuric acid at 90°C for 30 min. The tube was cooled on ice, after which, a 4 ml volume of 0.014 N H_2SO_4 was added with rapid mixing. Before analysis by HPLC, sample was diluted an additional 5 to 100 fold with 0.014 N H_2SO_4 containing 0.8 mg of adipic acid per ml as an internal standard and filtered through a 0.45 µm HAWP membrane filter (Millipore Corp., Bedford, Mass.) to remove particulate material. The injected sample volumes ranged from 10 to 50 µl. Sample was eluted with 0.014 N H_2SO_4 at a flow rate of 0.7 ml/min from Shimp-Pack CLC-ODS-M column (Schimadzu Corp. Japan) on a PerkinElmer series 200 HPLC comprising Series 200 auto-sampler, Series 200 pump, nd Series 200 UV/VIS detector Absorbance of crotonic acid was measured at 214 nm. The amount of crotonic acid produced from PHB was calculated from the regression equation derived from known crotonic acid standards.

RESULTS

Characteristics of PHB producing Bt isolate

Colonies of CMBL-BT-6 were stained with Sudan Black B solution for 10 min and those which

had absorbed stain turned blue and were taken as positive for PHB production (Fig. 1). CMBL-BT-6 formed 'pan cake' like circular colonies with smooth margins on nutrient agar.

CMBL-BT-6 isolates after Gram staining demonstrated Gram-positive rods.

Effect of different growth parameters on PHB yield

Incubation time

Figure 2 shows the effect of different incubation times on the production of PHB. The maximum yield of 0.89 g/L was obtained after 24 h incubation at 37°C. The yield after 144 h of incubation was 0.62g/L. Negative controls did not show any PHB production.

Temperature

Figure 3 shows the effect of different incubation temperatures on the production of PHB. The maximum yield of 1.02g/L was obtained after 24 h incubation at 37°C. The yield at 4°C was 0.0005g/L. All optimization experiments were carried out with suitable negative controls which did not show any PHB yield.

pH

Figure 4 shows the effect of different pH on the production of PHB. The maximum yield of 0.983g/L was obtained at pH 7.0 after 24 h incubation at 37°C. The yield at pH 2.0 was 0.015g/L.

Different carbon sources

Figure 5 shows the effect of different carbon sources on the production of PHB. The maximum yield of 0.867g/L was obtained from maltose after 24 h incubation at 37°C.

Different nitrogen sources

Figure 6 shows the effect of different nitrogen sources on the production of PHB. The maximum yield of 0.953g/L was obtained from ammonium sulphate after 24 h incubation at 37°C. The yield with tryptone under the similar growth conditions was 0.463g/L.

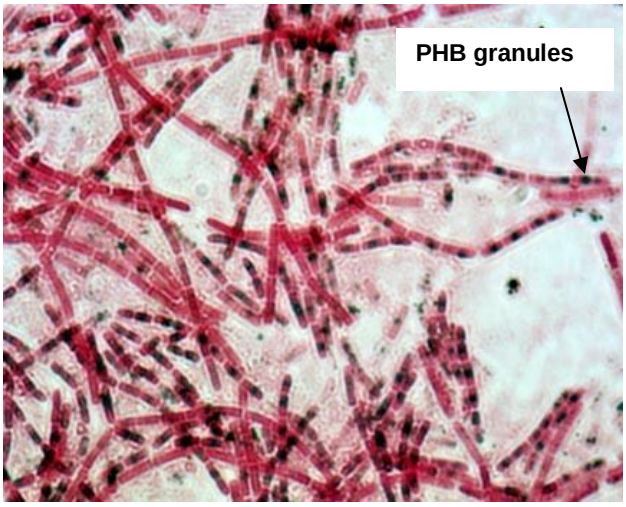

Fig. 1. Dark-blue granules of PHB inside cell.

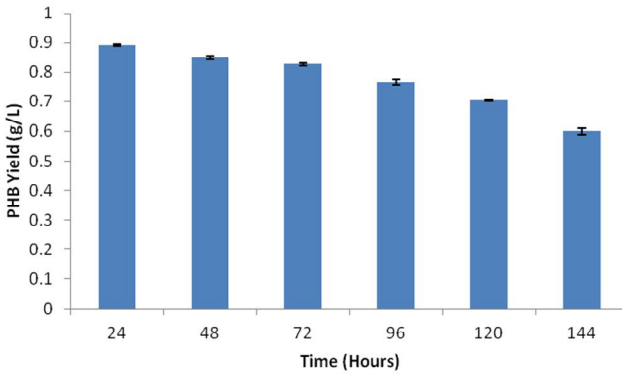

Fig. 2. PHB (g/L) produced by *Bacillus thuringiensis* strain CMBL-BT-6 incubated for different time periods at 37°C in nutrient broth (pH 7) containing 2% glucose as substrate as well as carbon source, and ammonium nitrate as nitrogen source .

Different substrate concentrations

Figure 7 shows the effect of different substrate concentrations on the production of PHB. The maximum yield of 0.953g/L was obtained with 3.0% of substrate after 24 h incubation at 37°C. A yield of 0.158g/L was obtained from 1.0% o substrate concentration.

PHB yield under optimized conditions

Under optimal culture conditions in nutrient broth (pH 7.0) containing 1% maltose as carbon

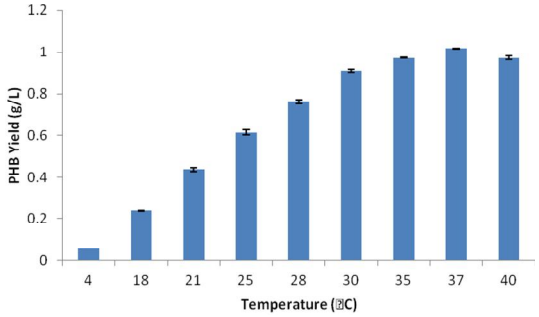

Fig. 3. PHB yield (g/L) produced by *Bacillus thuringiensis* strain CMBL-BT-6 incubated at different temperatures for 24 h in nutrient broth (pH 7) containing 2% glucose as substrate as well as carbon source, and 0.1% ammonium nitrate as nitrogen source.

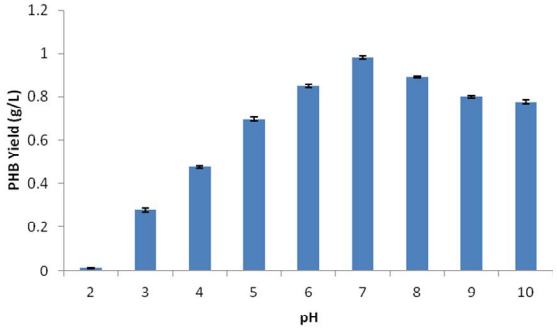

Fig 4. PHB yield (g/L) produced by *Bacillus thuringiensis* strain CMBL-BT-6 incubated at 37ºC for 24 h in nutrient broth at different pH, containing 2% glucose as substrate as well as carbon source, and 0.1% ammonium nitrate as nitrogen source.

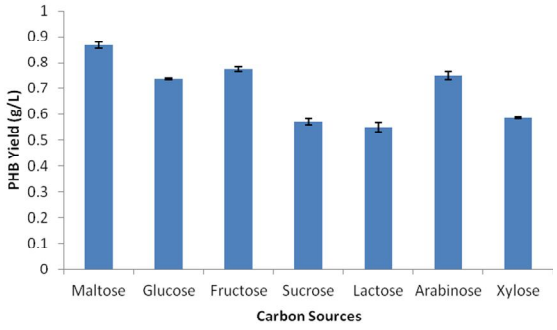

Fig. 5. Effect of different carbon sources (1%) on PHB yield (g/L) produced by *Bacillus thuringiensis* strain CMBL-BT-6 grown incubated 37ºC for 24 h in nutrient broth containing 2% glucose as substrate, and 0.1% ammonium nitrate as nitrogen source.

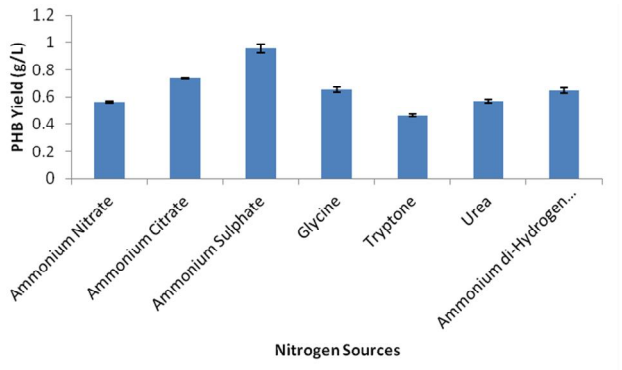

Fig. 6. Effect of different nitrogen sources (0.1%) on PHB yield (g/L) by CMBL-BT-6 strain incubated at 37ºC for 24 h in nutrient broth containing 2% glucose as substrate, and 1% maltose as carbon source.

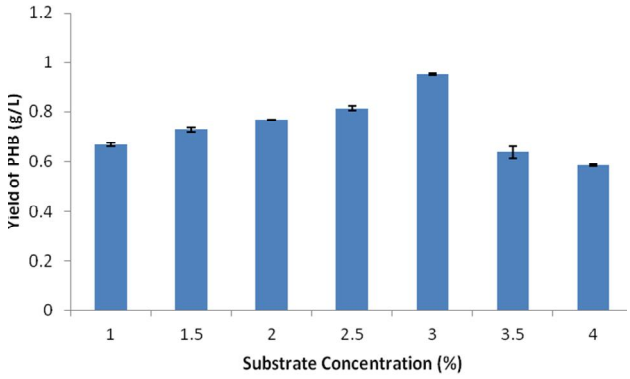

Fig. 7. `Effect of different substrate concentrations (%) on PHB yield (g/L) by CMBL-BT-6 strain incubated at 37ºC for 24 h in nutrient broth containing 0.1% ammonium sulphate as nitrogen source and 1% maltose as carbon source.

source, 0.1% ammonium sulphate as nitrogen source and 3.0% glucose as substrate, the strain CMBL-BT-6 produced 2.75g/L of PHB after incubation for 24 h at 37ºC. Figure 8 shows the purified form of PHB produced by CMBL-BT-6 strain under the above described growth conditions.

HPLC analysis

The HPLC chrmatogram indicated that the substance that has been produced by CMBL-BT-6 was PHB (Fig. 9).

Fig. 8. Purified form of polyhydroxybutyrate (PHB) produced by CMBL-BT-6 strain of *Bacillus thuringiensis* under optimized culture conditions in nutrient broth (pH 7) containing 3.0% glucose as substrate, 0.1% ammonium sulphate as nitrogen source and 1% maltose as carbon source incubated at 37ºC for 24 h.

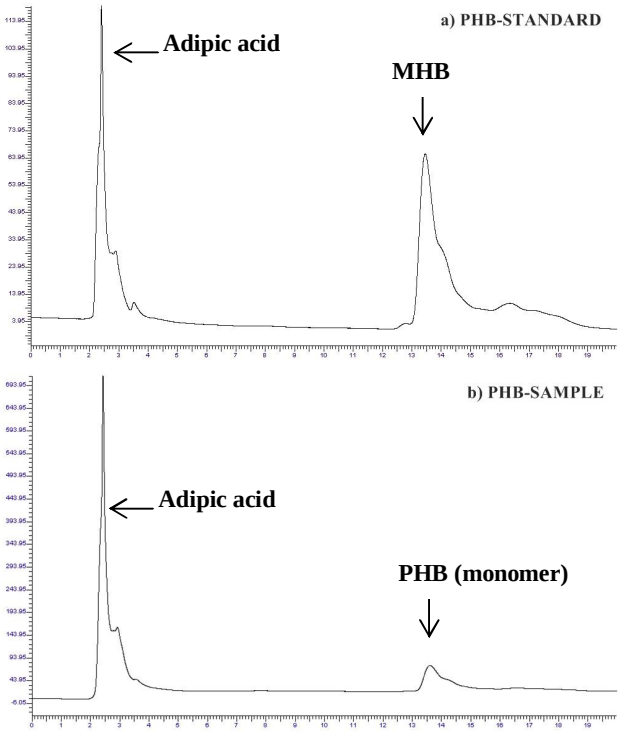

Fig. 9. Chromatograms of HPLC analysis: a, mono-hydroxybutyric acid (Standard); b, acid hydrolyzed poly-β-hydroxybutyric acid.

DISCUSSION

CMBL-BT-6 isolate has been shown to produce PHB when incubated at 37^{o}C for 24 h under optimized condition. pH 7.0 was found to be optimum for best production of PHB. This was in agreement with work of Aslim *et al*. (2002) who observed that the PHB in *Rhizobium* strain grown on yeast extract mannitol broth adjusted to pH 7.0, produced PHB 0.01 to 0.5g/L which is 1.38 and 40 percent of cell dry weight. Tavernler *et al*. (1997) also showed higher production of exopolysaccharide and PHB by *Rhizobium meliloti* at pH 7.0.

Amongst different carbon sources like glucose, fructose, sucrose, maltose, arabinose, lactose and xylose (1%) used in the nutrient medium, maltose was found to be the best carbon source. yielded the mean PHB of 0.867g/L. Working with different carbon sources in MSM broth (pH 7.0), Khanna and Srivastava (2005b) observed higher PHB yield in the presence of fructose by *Alcaligenes eutrophus*. They reported that glucose and fructose, being monosaccharides were readily utilized by bacteria and, hence, have supported growth and subsequently PHB production. The complex molecules like starch and lactose were not utilized. Yuksekdag *et al*. (2003) reported that the highest PHB synthesis was found in *Bacillus subtilis* and *Bacillus megaterium* when glucose was used as the carbon source in YEM medium. The production of PHB in *B. megaterium* was studied by Hori *et al*. (2002) and reported highest production of PHB contents when glucose was used. In our experiment, CMBL-BT-6 has given highest PHB yield on maltose which is a complex carbon source. As the complexity of the carbon source has increased, PHB yield also increased.

Ammonium sulphate was found to be the best nitrogen source for PHB production by CMBL-BT-6. Nutrient limitation is necessary to trigger PHB accumulation, and generally ammonia is used as the critical control factor for uncoupling the growth of cells and PHB production (Wang and Lee, 1997). A recombinant *E. coli* strain gave the maximum PHB content (about 60% PHB of DCW) at a specific combination of yeast extract and peptone (Mahishi *et al*., 2003). These results are in agreement with the

results obtained by Khanna and Srivastava (2005a) who also reported highest PHB production (2.26g $100ml^{-1}$) by *Ralstonia eutropha* in MSM medium supplemented with ammonium sulphate. Mercan *et al*. (2002) have reported in two strains of *Rhizobium* sp. less production of PHB in yeast exract mannitol (YEM) broth medium (pH 7.0) with different carbon (glucose, sucrose, arabinose) and nitrogen (L-cysteine, L-glycine, DL-tryptophan, protease peptone, potassium nitrate) sources, while the highest level of PHB accumulation was observed in the medium with L-cysteine and L-glycine.

Table I.- Comparison of PHB yield produced by different microorganism under different growth conditions.

Bacterial strain	Growth medium	PHB content	Reference
Rhizobium spp.	Yeast extract mannitol (pH 7.0)	0.01 to 0.5g/L	Aslim *et al.* (2002)
Alcaligenes eutrophus	Mineral Salt Medium+Fructose (pH 7.0)	2.260g $100ml^{-1}$	Khanna and Srivastava (2005a)
Rhizobium spp.	Yeast Exract Mannitol+	High Yield	Mercan *et al.* (2002)
Azotobacter chroococcum	L-cysteine, L-glycine (pH 7.0)	High Yield	PalSaha and Paul (2003, 2005)
Rhizobium meliloti	N2-free stock dale medium+2% Glucose (pH 7.0)	High Yield	Tavernler *et al.* (1997)
Bacillus subtilis	Mineral Salt Medium (pH 7.0)	High Yield	Yuksekdag *et al.* (2003)
Bacillus thuringiensis strain CMBL-BT-6	Nutrient broth (pH 7), 3.0% glucose, 0.1% ammonium sulphate, 1% maltose at 37ºC for 24 h	2.75 g/L	This study

Glucose has been used as a substrate for PHB production. Different concentrations of substrate have been used. It has been found out that 4% of substrate (glucose) is optimum for maximum production of PHB which gives 0.146g/L. PalSaha and Paul (2003, 2005) observed that *Azotobacter chroococcum* MAL-201, accumulated PHB. The polymer yield accounted to 69 % of cell dry weight when grown in N_2-free stock dale medium (pH 7.0) containing 2 % (w/w) glucose as substrate. Table I shows comparison of PHB production by different microorganism under different growth conditions.

HPLC - UV detection, provides a simple technique for routine analysis of PHB. It provides the advantages of (i) fractionation of materials in the digest before detection of crotonic acid, (ii) greater sensitivity and accuracy across a wide concentration range, and (iii) easy and rapid sample analysis.

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