Production of the biopolymer – Polyhydroxybutyrate (PHB) using *Bacillus subtilis*

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Abstract

Polyhydroxybutyrate (PHB) is a very basic polymer which is accumulated as energy reserve material by many bacteria. It is water soluble, biocompatible and non-toxic. A study was conducted to isolate and evaluate the PHB producing soil bacteria. A total number of 7 different bacterial genera were isolated and the amounts of PHB were estimated. *Bacillus subtilis* was the most efficient producer among the test isolates. Further, 25 isolates of *Bacillus* sp. were isolated and 18 of which were identified to be *Bacillus subtilis.* Among them, two isolates (isolate nos. 15 & 25) were confirmed to accumulate maximum PHB and *Bacillus subtilis* isolate no. 25 produced more PHB with different sugars, organic and inorganic nitrogen sources at varying concentrations.

Keywords : *Bacillus subtilis,* biocompatibility, biopolymer, organic nitrogen, polyhydroxybutyrate, sugars

INTRODUCTION

Synthetic polymers such as nylon, polyethylene, and polyurethane have raised a number of environmental and human health concerns because they are non-biodegradable and are derived mostly from non-renewable resources. Due to their durability and strength, synthetic polymers are more persistent and so their disposal is extremely complex (Fietchler, 1990). These problems have focused increased awareness on polymers that are biodegradable and are derived from natural or biological systems popularly called biopolymers.

The biopolymers are produced from biological precursors and are enormously available in plants, animals and most specifically from microorganisms. Some of the important biopolymers are polysters (polyhydroxyalkonates, polyhydroxybutyrates, polyhydroxybutyrate-hydroxyvalerate), proteins (collagen or gelatin, elastin, adhesives), polysaccharides (xanthan, dextran, levan, curd lan) (Muller and Seebach, 1993). Polyhydroxybutyrate (PHB) is one of the important biopolymers produced by microorganisms. It is an intracellular inclusion, which serves as a lipid storage material (Martin *et al*., 2001). Such biopolymers may prove to have a variety of environmental benefits and significant applications due to their biodegradability and biocompatibility. The present paper deals with soil bacteria and their role in the production of PHB at different growth conditions.

MATERIALS AND METHODS

Rhizosphere soil samples were plated at appropriate dilutions on to nutrient agar medium to isolate *Bacillus subtilis*, *B. megaterium*, *Pseudomonas* sp. etc., and on Jensons's medium to isolate *Azotobacter beijerinckii*. All the organisms were further confirmed using conventional microbiological methods such as Gram's staining, culture on selective media and biochemical tests based on standard protocols. Then the isolates were subjected to preliminary screening for the production of PHB on nile blue agar (Kranz *et al*., 1997). Further, the isolates were cultivated using broth media and PHB was extracted and estimated (Sheldon *et al*., 2002).

About 25 soil samples were collected from the surrounding areas of Dr. G.R.D College of Science, Coimbatore, Tamilnadu, South India, and were subjected to *Bacillus* sp. isolation. Microscopic examination, culture studies and biochemical tests were done to confirm the isolates of *B. subtilis*. Subsequently, polyhydroxybutyrate (PHB) was extracted from all the *B. subtilis* isolates and estimated using standard procedure (Sheldon *et al*., 2002). Two best PHB producers were selected and were subjected to further studies. The two selected *B. subtilis* isolates were grown in media with different sugars (glucose, sucrose, mannitol, and commercial sugar like jaggery) at vary-

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ing concentrations (1%, 1.5%, 2%, 2.5% & 3%) and were estimated for PHB production. The selected *B. subtilis,* isolates were also grown in media with organic nitrogen (protease peptone) at varying concentrations (1%, 1.5%, 2%, 2.5% & 3%) and with inorganic nitrogen (potassium nitrate) at concentrations of 5 mM, 10 mM, 15 mM, 20 mM, & 25 mM and the rate of PHB accumulation was determined. The isolates were also allowed to grow for varying time periods (24 hrs, 48hrs, 72 hrs & 96 hrs) and were estimated for PHB production.

RESULT AND DISCUSSION

Totally 7 different soil bacteria such as *A. beijerinckii*, *Rhizobium* sp., *Pseudomonas* sp., *E. coli, Klebsiella* sp., *B. subtilis* and *B. megaterium* were isolated from the soil in and around Dr. G.R.D College of Science, Coimbatore, Tamilnadu and confirmed for PHB production. Among the seven soil bacteria, *B. subtilis* produced maximum amount (4.6µg/ml) of PHB (Figure 1). Earlier Aslim *et al.* (2002) have reported 27 isolates of *Bacillus* were able to produce PHB. However, Anderson and Dawis, (1997) reported that *Alcaligens, Azotobacter* and *Pseudomonas* produced maximum amount of PHB and Ugur *et al*. (2002) have reported *Streptomyces* as a high PHB producer. Thus, this study is in line with the report of Aslim *et al.* (2002) and confirms that *B. subtilis* is one of the potential PHB producers.

B. subtilis was considered for further analysis and a total number of 25 isolates of *Bacillus* sp. were isolated from 25 different soil samples. Among the 25 isolates of *Bacillus* sp., 18 were confirmed to be *B. subtilis* and the rest 7 as *B. megaterium* based on microscopic, culture and biochemical tests (Table 1). The number of isolates obtained in the present study is significant when compared to 29 & 24 isolates of *B. megaterium* and *B. subtilis* isolates obtained respectively, from 306 soil samples subjected by Waksman (1961). When all the 18 *B. subtilis* isolates were further analyzed for PHB production, the isolate nos. 15 and 25 were found to produce as much as 8.1 µg and 10.5 µg of PHB per ml of culture, respectively (Fig. 2). Effects of carbon and nitrogen sources over the production of PHB by the *B. subtilis* isolates nos. 15 & 25 are given in tables 2 & 3. Among the various carbon sources, glucose was found to support maximum growth and PHB production (Table 2). At 3% glucose concentration, *B. subtilis* isolate no. 25 produced as much as 9.5 µg of PHB per ml of culture. The effect of other sugars, such as sucrose, mannitol, and commercial sugar (jaggery) over polyhydroxybutyrate storage was not remarkable.

The effects of different concentrations of organic nitrogen (protease peptone) and inorganic nitrogen (potassium nitrate) on the production of PHB by the soil *B. subtilis* isolate nos. 15 & 25, is presented in table 3. When protease peptone was used as nitrogen source at 2.5% the yield was maximum as a concentration of 10.5 µg of PHB per ml of culture was obtained in *B. subtilis* isolate no. 25 (Table 2). Potassium nitrate (KNO_3), when used as nitrogen source, at 25 mM, as much as

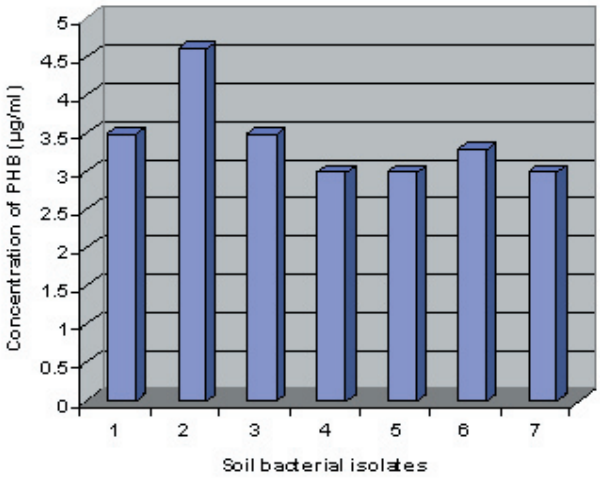

Figure 1. Production of PHB by different species of soil bacteria
1. Azotobacter beijerinckki; 2. *B. subtilis;* 3. *B. megatrium;* 4. *E. coli;* 5. *Rhizobium sp.*; 6. *Pseudomonas* sp.; 7. *Klebsiella* sp.

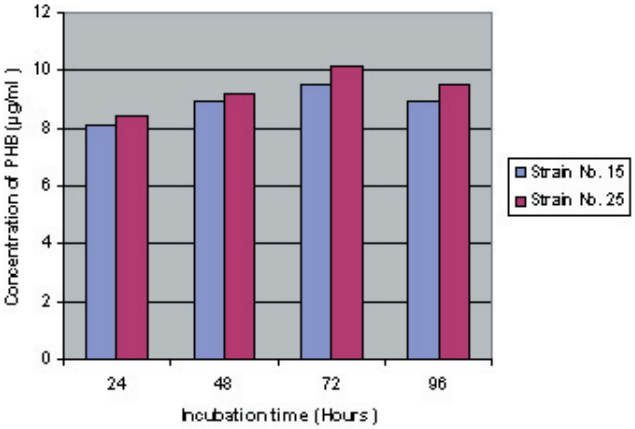

Figure 2. Production of PHB by the isolates of *B. subtilis* under different periods of incubation

Table 1. Microscopic and biochemical test results of *Bacillus* species obtained from the soils of the present study area.

Test	*B. subtilis*	*B. megaterium*
Gram's staining	+	+
Motility	-	-
Rhizoid growth	-	-
Protein toxic crystals	-	-
Spore staining	-	-
Glucose fermentation	A	A
Vogus Proskour	+	-
Citrate utilization	+	+
Catalase	+	+
Nitrate	+	+

A – Acid Production (+) - Positive (-) Negative

Table 2. Effects of different carbon sources on the production of PHB by the isolates of *B. subtilis*. Values are amounts of PHB (µg/ml of culture) produced in different concentrations of sugars

S. No.	Type of Sugar	Isolate number	Concentration of sugars (%)				
			1.0	1.5	2.0	2.5	3.0
1.	Glucose	15	7.55	7.8	8.7	9.0	9.05
		25	7.6	8.0	9.0	9.2	9.5
2.	Sucrose	15	8.0	8.02	8.01	8.03	8.2
		25	8.01	8.03	8.04	8.05	8.4
3.	Mannitol	15	8.0	8.0	8.05	8.03	8.04
		25	8.0	8.0	8.01	8.05	8.06
4.	Jaggery	15	8.0	8.0	8.02	8.02	8.04
		25	8.0	8.01	8.03	8.03	8.05

Table 3. Effect of potassium nitrate and protease peptone on the production of PHB by *B. subtilis*. Values amounts of PHB (µg/ml of culture) produced in different concentrations of inorganic and organic nitrogen sources

S.No.	Isolate number	Type of N_2 source										
		Inorganic N_2 – Potassium nitrate						Organic N_2– Protease peptone				
		5mM	10mM	15mM	20mM	25mM	2%	1%	1.5%	2%	2.5%	3%
1	15	7.0	7.0	7.4	7.8	8.1	5.4	8.4	8.7	9.0	9.5	8.0
2	25	7.0	7.4	8.0	8.1	8.2	6.7	9.8	10.0	10.2	10.5	9.2

8.2 µg of PHB/ml was produced with the soil *B. subtilis* isolate no. 25, but at 2% the PHB production was low. Yuksekdag *et al.* (2004), have reported highest yields of PHB in the medium with protease peptone as nitrogen source in 25 *B. subtilis* (78.69%) and in 12 *B. megaterium* (77%). Page (1992) tested PHB production with a variety of commercially available complex nitrogen sources such as fish peptone, protease peptone, yeast extract, casilone, phyton, and tryptone and found that the complex nitrogen sources increased the yields of PHB produced by *A. vinelandi* UWD strain. The effects of different nitrogen and carbon sources on PHB production was studied in two isolates of *Rhizobium* sp, by Mercan *et al.* (2002) and they have reported that the isolates produced less PHB in Yeast Extract Mannitol Agar (YEMA) broth medium with different carbon (glucose, sucrose, arabinose) and nitrogen (L-glycine, DL-tryptophan, L-cysteine, protease peptone, potassium nitrate) sources, while the highest level of PHB was observed in the media with L-glycine and L-cysteine.

Incubation time was also a factor in determining the amount of PHB production by the test *B. subtilis* isolate nos. 15 & 25. The impacts of varying incubation time on the production of PHB by the test isolates is shown in figure 2. It was found that PHB production was maximum at 72 hours (10.2 µg of PHB/ml) with isolate

no. 25. However, at 96 hour, the PHB accumulation was found to decline (9.5 µg of PHB/ml of culture).

Thus, this study concludes that *B. subtilis* is one of the potential PHB producing microorganisms and the optimum production of PHB could be obtained in the medium containing glucose as carbon source, protease peptone as nitrogen source and at 72 hours of incubation time.

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