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## Efficient poly- $\beta$ -hydroxybutyrate production from *Bacillus* sp.

**Nisha Verma, SS Sindhu and Sneh Goyal**

### Abstract

Poly-hydroxyalkanoates are biodegradable polymers produced by many microorganisms to overcome environmental stress conditions. For production of biodegradable plastic polymers at low cost, one hundred and fifteen Gram positive bacterial isolates were obtained from soils amended with sewage sludge. Different bacterial isolates were screened for production of poly- $\beta$ -hydroxybutyrate (PHB) using Nile blue A and Sudan black B dyes. Only 10.65% of the total *Bacillus* isolates showed PHB production. Four *Bacillus* strains showed high fluorescence with Nile blue A and accumulated PHB granules in the cell. *Bacillus* strain Sld110 produced 38.6% PHB under shaking and 52.2% under stationary conditions using 1% sewage sludge as carbon substrate. Thus, the exploitation of these *Bacillus* strains for production of biodegradable plastics by utilizing sewage sludge waste will solve the problems associated with waste disposal and also reduce environmental pollution.

**Keywords:** poly- $\beta$ -hydroxybutyrate, *Bacillus* strains, sewage sludge, Nile blue A, Sudan black B, biodegradable plastics, environmental pollution

### Introduction

The extensive use of synthetic petrochemical based plastics by industries and households led to its accumulation in the environment due to its non-degradable nature. This accumulation has caused waste disposal problems leading to alteration of the native biodiversity and environmental pollution (Full *et al.*, 2006) [9]. Thus, concept of biodegradable plastics emerged as a solution for this problem when a wide variety of poly- $\beta$ -hydroxyalkanoates (PHA) natural biopolymers were found as an intracellular storage compound in different taxonomic group of prokaryotes (Anderson and Dawes, 1990) [1]. The homopolymer poly- $\beta$ -hydroxybutyrate (PHB) is the best known example of the PHA family and prokaryotic organisms are known to produce PHB amounting to as much as 80% of their cellular weight (Doi, 1990) [8]. These PHB biopolymers are biodegradable and biocompatible thermoplastics, synthesized by cells under growth limiting conditions (Bertrand *et al.*, 1990; Vijayendra *et al.*, 2007) [3, 29]. PHB is accumulated as an energy reserve material by various microorganisms belonging to the genera *Alcaligenes*, *Azotobacter*, *Bacillus*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, *Staphylococcus* and *Rhodococcus* (Pierce and Schroth, 1994; Chen and Page, 1997; Singh *et al.*, 2009; Joshi and Jaysawal, 2010) [20, 5, 25, 12].

PHB and its copolymer based plastics have been produced on industrial scale and have potential applications in many consumer products, medical, agricultural and industrial fields, because the polymers can be made into biodegradable razors, shampoo bottles, wrapping films, bags, and sheets etc. (Lee, 1996; Chen and Wu, 2005; Arun *et al.*, 2006) [16, 6, 2]. However, the high cost of the manufacturing process of this biodegradable plastic is attributed to the cost of carbon source, fermentation process and the downstream processing (Choi and Lee, 1999) [7]. About 50% of the production costs of the PHA are added by the cost of carbon source (Halami, 2007) [11]. Therefore, the use of inexpensive and renewable carbon substrates viz. agroindustrial waste or sewage sludge as PHA feedstock can contribute to as much as 40-50% reduction in the overall production cost (Kim, 2000; Khardenavis *et al.*, 2005; Ramadas *et al.*, 2009) [14, 13, 21]. Moreover, the use of Gram positive bacteria such as *Bacillus* sp. are ideal candidates for industrial scale PHB production due to lack of lipopolysaccharide layer and their ability to grow rapidly on low cost agro-industrial wastes (Valappil *et al.*, 2007a; Santimano *et al.*, 2009) [27, 23].

In this study, *Bacillus* isolates obtained from sewage sludge amended soils were tested for PHB production under laboratory conditions. Selected strain Sld110 was tested for the

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of PHB using sewage sludge as carbon substrate to minimize the cost for the production of biodegradable plastics.

## Materials and Methods

### Isolation of *Bacillus* strains

Soil samples were collected from sewage sludge amended soils of different parts of Haryana in Northern India. Ten grams of soil from each composite soil sample was transferred into 90 ml sterilized water in 250 ml-capacity Erlenmeyer flask and shaken vigorously. The soil suspension was heated at 80°C in a water bath for 30 minutes to kill the vegetative cells and only spore formers were left. The soil suspension after cooling was serially diluted upto  $10^{-5}$  and 0.1 ml of  $10^{-4}$  and  $10^{-5}$  dilutions was spread on nutrient agar medium plates. The medium plates were incubated for 2-3 days at  $28 \pm 2^\circ\text{C}$ . The bacterial colonies with different morphologies were picked up, purified and maintained on nutrient agar medium slants at 4°C in a refrigerator. Gram staining, spore staining and biochemical tests of the bacterial strains were performed to identify the bacteria.

### Screening of *Bacillus* strains for PHB production

The qualitative screening of bacterial isolates for PHB production was done by using Sudan black B staining method (Schlegel *et al.*, 1970) [24] and Nile blue A method (Ostle and Holt, 1982) [17]. In Sudan black B method, *Bacillus* strains were stained with Sudan black B (0.3%, w/v) and counter stained with aqueous safranin (0.5%, w/v). The PHB granules appeared as blue black droplets and cytoplasmic part of microorganisms appeared as pink under oil immersion objective lens.

In Nile blue A method, bacterial strains were spotted on minimal medium plates containing Nile blue (0.05%, w/v) and the plates were incubated at  $28 \pm 2^\circ\text{C}$  in a BOD incubator for 15 days. The plates were examined for fluorescence under ultraviolet trans-illuminator at different days of growth. The PHB producing bacterial strains showed fluorescence and the level of fluorescence in different strains was used as indicator for the amount of PHB produced by the bacteria. Four bacterial isolates i.e. Sld64, Sld99, Sld101 and Sld110, which showed maximum number of PHB droplets and higher fluorescence as compared to other isolates, were selected for further studies.

### Quantitative estimation of PHB

PHB was estimated by using method of Law and Slepecky (1961) [15] and the amount of PHB produced was calculated from the standard curve prepared by using poly- $\beta$ -hydroxybutyrate. Twenty five ml of bacterial culture was centrifuged for 20 min at 8,000 rpm at 4°C to sediment the cells. Pellet of the cells was washed with double distilled water and mixed thoroughly in 5 ml of sodium hypochlorite by vortexing it. This mixture was incubated at 37°C for 1 h and then centrifuged at 8,000 rpm for 20 minutes to sediment the lipid granules. The solid pellet obtained after decanting of supernatant was resuspended in 5 ml of distilled water and centrifuged at 8,000 rpm for 20 minutes. The supernatant was decanted again and the pellet adhering to walls of centrifuge tube was washed successively with 5 ml of acetone, diethyl ether and ethanol to remove water which interferes with the extraction of polymer.

The pellet was suspended in 5 ml of acetone by vortexing and centrifuged at 8,000 rpm for 20 minutes. The supernatant was decanted and the sediment was suspended in 5 ml of diethyl

ether by using vortex and centrifuged at 8,000 rpm at 4°C for 20 minutes. Then, pellet for granules was dissolved in chloroform in boiling water bath for 2 minutes. After cooling the solution, it was centrifuged at 8,000 rpm for 20 minutes at room temperature. The supernatant was saved in 10 ml graduated test tube and sediment was re-extracted twice with 3 ml of chloroform at 100°C for 2 minutes. The whole solution was filtered through Whatman No. 1 filter paper (previously treated with hot chloroform). The chloroform extracts were pooled and made to 10 ml with chloroform in clean glass tubes. Chloroform extracts were heated in boiling water bath until all the chloroform gets evaporated. To this, 10 ml of concentrated  $\text{H}_2\text{SO}_4$  was added and tubes were capped with glass marbles. Then tubes were heated for 20 minutes in boiling water bath, cooled and mixed thoroughly. Absorbance of the solution was read at 235 nm against a concentrated  $\text{H}_2\text{SO}_4$  blank on UV-VIS spectrophotometer (Sigma-Aldrich Techware).

### Measurement of bacterial growth

Bacterial growth was estimated in the minimal medium containing 1% carbon source in the form of sucrose and sewage sludge. Dry weight of the cells was measured at different days of incubation. Twenty-five ml of culture broth was centrifuged at 10,000 rpm at 4°C for 15 min and the supernatant was discarded. The sedimented cell pellet was transferred to pre-weighed aluminium foil cup and kept at 80°C in an oven for 2 h and weighed. The amount of dry cells per litre of broth was calculated.

### PHB production using sewage sludge as a carbon substrate and standardization of conditions for PHB production

Optimization of cultural conditions for maximum PHB production was carried out using the PHB producing *Bacillus* isolates Sld64, Sld99, Sld101 and Sld110. Minimal medium was inoculated with 1% inoculum that had been pre-grown for 24 h in the nutrient broth and incubated at  $28 \pm 2^\circ\text{C}$  under different growth conditions both under stationary as well as shaking conditions on an orbital shaking incubator at 120 rpm.

Sewage sludge (1%) was used as a carbon source for PHB production using selected bacterial strains. Bacterial growth and PHB production were monitored at different time intervals ranging from 24 to 72 h in 150 ml-capacity Erlenmeyer flasks at  $28 \pm 2^\circ\text{C}$  to know maximum yield of PHB under both shaking and stationary conditions.

## Results

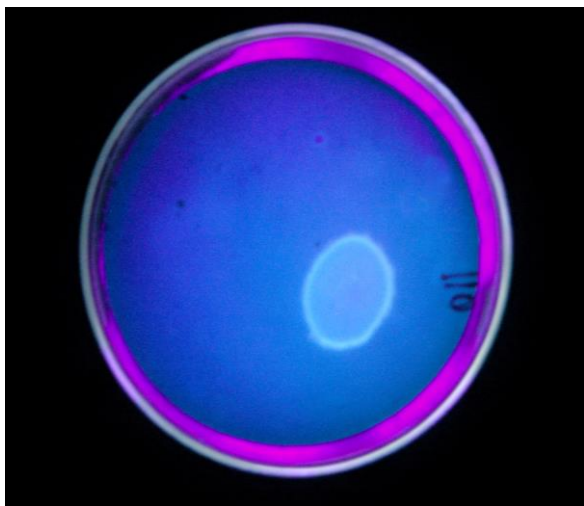
A total of 115 bacterial isolates were obtained from sewage sludge amended soils on nutrient agar plates. The bacterial colonies which varied in shape, size and appearance were picked up, purified by streaking and transferred on nutrient agar medium slants. Bacterial colonies varied from small to large in size, round to irregular in shape and were cream color in appearance.

### Screening of bacterial isolates for PHB production

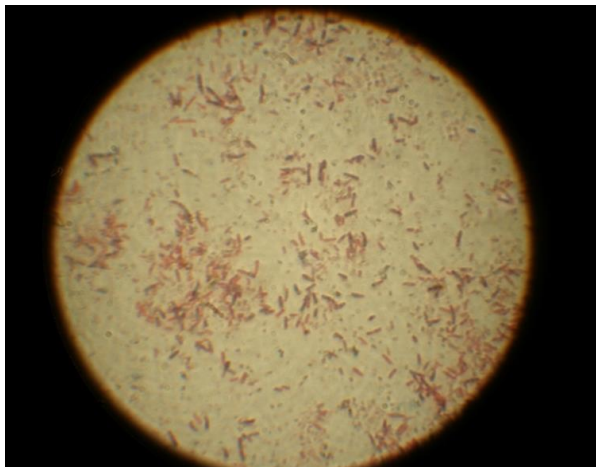
The purified bacterial isolates were screened for PHB production using Nile blue. Variation in the level of fluorescence was observed in different bacterial isolates and only nineteen *Bacillus* isolates showed PHB production (Fig. 1). Four *Bacillus* isolates, which showed maximum fluorescence, were further selected for PHB production

studies (Table 1). Two bacterial isolates Sld64 and Sld110 produced more fluorescence as compared to isolates Sld99 and Sld101 after 10 days of incubation.

Bacterial isolates which gave significant fluorescence with Nile blue A dye were further verified for PHB production using Sudan black B method. Four *Bacillus* strains also produced large amount of PHB granules which appeared as blue black droplets in the cells when stained with Sudan black B dye. *Bacillus* isolates Sld101 and Sld110 showed 3 and 4 number of black granules, respectively (Fig. 2). The isolate Sld64 showed two black granules whereas Sld99 showed only one black granule.



**Fig 1:** *Bacillus* isolate Sld110 showing fluorescence with Nile blue A dye.



**Fig 2:** *Bacillus* isolate Sld110 showing black granules after staining with Sudan black B.

#### PHB production using sucrose as C source

For quantitative estimation of PHB or PHAs, different methods have been reported by various workers using chloroform, sodium hypochlorite, dispersion with chloroform and sodium hypochlorite but Law and Slepecky (1961) [15] method is widely used for quantitative estimation of PHB. In this method, PHB is converted to crotonic acids by heating with concentrated  $H_2SO_4$ . As this method is highly specific for PHB estimation, so it was used for quantitative estimation of PHB production by different *Bacillus* strains during the present study.

PHB accumulation as an internal reserve of carbon and energy takes place under nutrient limiting conditions during

late log and stationary phase of growth. PHB production by selected four isolates in the minimal medium containing sucrose as carbon source is shown in Table 2. Bacterial isolate Sld110 produced highest amount of PHB i.e., 29.2, 29.3 and 25.2% at 24, 48 and 72 h, respectively using sucrose as carbon source under stationary conditions at  $28 \pm 2^\circ C$ . Another *Bacillus* isolate Sld101 also showed significant production of PHB at 24 and 72 h of growth. The dry weight of all the four *Bacillus* strains increased linearly upto 72 h of growth and dry weight of cells was found more under shaking conditions than under stationary conditions. The amount of PHB produced under shaking conditions was found related to the growth phase of bacteria as it was produced less in initial log phase of growth and was produced in maximum amount after 48 h.

#### PHB production using sewage sludge as C substrate

In the minimal medium broth containing sewage sludge as carbon source, maximum cell biomass (493 mg/l) was observed at 48 h of growth under shaking conditions (Table 3). PHB production was found more under stationary conditions than shaking conditions at different times of incubation using sewage sludge as carbon source. Bacterial isolate Sld110 produced highest amount of PHB (52.2%) after 48 h using sewage sludge as carbon source under stationary conditions at  $28 \pm 2^\circ C$ .

#### Discussion

Poly- $\beta$ -hydroxybutyrate producing bacteria were isolated from soils amended with sewage sludge using nutrient agar medium. Screening of PHB producing *Bacillus* strains was done by using Nile blue A dye and fluorescence of PHB producing bacteria was observed under UV transilluminator (Fig. 1). Only 10.65% isolates out of 115 tested, showed fluorescence indicating that only limited *Bacillus* strains possess the ability of PHB production. Similarly, production of PHB have been reported by different *Bacillus* species i.e., *B. megaterium* (Reddy *et al.*, 2009; Pandian *et al.*, 2010) [22] [19], *B. subtilis* (Yuksekdag *et al.*, 2004; Singh *et al.*, 2009) [30] [25] and *Bacillus* sp. (Santimano *et al.*, 2009; Joshi and Jaysawal, 2010) [23, 12]. Pierce and Schroth (1994) [20] also screened *Pseudomonas* colonies which accumulated poly- $\beta$ -hydroxybutyrate on Nile blue A incorporated medium.

The PHB production by these *Bacillus* strains in this study was further verified by the presence of blue black droplets inside the pink cells using Sudan black B (Table 1). Four *Bacillus* strains were found to produce large amount of PHB granules which appeared as blue black droplets in the cells when stained with Sudan black B dye. Gerhardt *et al.* (1981) [10] used Sudan black B to indicate the contents of fatty substance and lipid inclusions in bacteria. Stained PHB granules appeared as blue black droplets inside the pink colored cells after 24 h of growth. Page and Cornish (1993) [18] also observed PHB inclusions in *Azotobacter vinelandii* at 18 to 24 h of growth using Sudan black B dye. Similarly, Joshi and Jaysawal (2010) [12] used Sudan black staining for PHB production and reported that nine bacterial isolates obtained from sewage sample of industrial area of Vapi and domestic sewage from Thane produced poly- $\beta$ -hydroxyalkanoate. Maximum production of PHA was showed by *Staphylococcus* species (64.67%) and *Bacillus* species (68.85%) from industrial and domestic sewage, respectively.

The *Bacillus* strain Sld110 started producing PHB within 24 h and thereafter, it declined after 84 h. It produced highest

amount of PHB i.e., 23.9% under shaking and 29.3% under stationary conditions using sucrose as carbon source (Table 2). Valappil *et al.* (2007b) [28] also reported that *Bacillus cereus* strain SPV produced PHB at a concentration of 38% of its dry cell weight in shaken flask cultures, using glucose as the main carbon source. Polymer production was then scaled up to 20 L batch fermentations where 29% dry cell weight of PHB was obtained within 48 h.

*Bacillus* strain Sld110 produced 38.6% PHB under shaking conditions and 52.2% under stationary conditions (Table 3). PHB production increased linearly during log phase of growth i.e., upto 72 h in minimal medium broth using sewage sludge as a carbon source and then declined under both growth conditions (data not shown). Borah *et al.* (2002) [4] found sucrose to be the best carbon source in comparison to glucose and fructose for cell growth and PHB accumulation by *Bacillus* strain RLj B-017. Efficient polyhydroxybutyrate production from *Bacillus thuringiensis* using sugarcane juice substrate (Thammasittirong *et al.* 2017) [26]. Yuksekdag *et al.* (2004) [30] reported that *Bacillus subtilis* and *B. megaterium* produced PHB in nutrient broth at different incubation times (between 6 and 48 h) and the production was found 0.101 and 0.142 g/l respectively. The percentage yields were 18.03 and 14.79 after 45 h of growth. In this study, *Bacillus* strain Sld110 produced highest cell biomass after 72 h of growth but it declined later under both shaking and stationary conditions.

Vijayendra *et al.* (2007) [29] also found that *Bacillus* sp. strain CFR256 produced 8.20 g/l PHB (51.2% dry cell biomass) after 72 h of fermentation. The production of PHB was less under shaking than stationary conditions at all different temperature and growth periods and it varied with different carbon sources. Similar results were reported by Chen and Page (1997) [5] who observed decreased PHB production by high aeration of beet molasses medium using *Azotobacter vinelandii*. Thus, more production of PHB under stationary conditions indicated that it is produced more under oxygen stress conditions. Pandian *et al.* (2010) [19] also reported that dissolved oxygen is a major limiting factor that affected PHB synthesis by *B. megaterium* during utilization of dairy waste and sea water.

Ramadas *et al.* (2009) [21] studied the production of PHB by *Bacillus sphaericus* strain NCIM5149 using different agroindustrial residues as carbon source. Jackfruit seed powder was found to give maximum production of PHB (19%) under submerged fermentation. The results presented in this study suggested that *Bacillus* strains Sld110 isolated from sewage sludge amended soils could be exploited for industrial production of PHB. Furthermore, the use of sewage sludge as carbon source can reduce the production cost of PHB for commercial production and will also solve the problems associated with waste disposal.

**Table 1:** Characteristics of different bacterial isolates isolated from various sewage sludge amended soils

Isolate no.	Morphology of colony	Gram reaction	Size of rods	Sporulation	Fluorescence	No. of black granules by Sudan black
Sld64	Round	+	medium	+	+++	2
Sld99	Irregular	+	medium	+	++	1
Sld101	Round	+	small	+	++	3
Sld110	Round	+	medium	+	+++	4

+ Low fluorescence  
 ++ medium fluorescence  
 +++ High fluorescence

**Table 2:** PHB production by different bacterial isolates in minimal medium using sucrose as a carbon source.

Incubation time (h)	Stationary conditions				Shaking conditions		
	Isolate no.	Dry weight of cells (mg/l)	PHB produced (mg/g dry weight of cells)	% PHB production	Dry weight of cells (mg/l)	PHB produced (mg/g dry weight of cells)	% PHB production
24	Sld64	615	160	26.0	632	103	16.2
	Sld99	613	159	25.9	624	100	16.0
	Sld101	583	168	28.8	596	99	16.6
	Sld110	600	175	29.16	615	147	23.9
48	Sld64	792	177	22.3	764	138	18.1
	Sld99	775	190	24.5	758	136	17.9
	Sld101	772	188	24.4	732	125	17.1
	Sld110	706	207	29.3	715	156	21.8
72	Sld64	1082	197	18.2	1220	139	11.4
	Sld99	1070	191	17.9	1379	145	10.5
	Sld101	1012	194	19.2	1058	141	13.3
	Sld110	1068	269	25.2	1337	169	12.6
CD at 5% level		0.9	18	0.79	31	12	43

**Table 3:** PHB production by bacterial isolate Sld110 in minimal medium using sewage sludge as a carbon source.

Incubation time (h)	Stationary conditions			Shaking conditions		
	Dry wt. of cells (mg/L)	PHB (mg/g dry wt. of cells)	% PHB production	Dry wt. of cells (mg/L)	PHB (mg/g dry wt. of cells)	% PHB production
24	270	115	42.6	344	79	23.0
48	353	145	41.1	493	83	17.0
72	188	98	52.2	212	82	38.6
C.D. at 5% level	17	9	2.1	22	7	1.5

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