

## Isolation and Identification of Some *Bacillus* spp. Producing Poly-3-hydroxybutyrate (PHB)

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A total of 240 strains of genus *Bacillus* were isolated from different samples collected at Hyderabad, India. Poly-3-hydroxybutyrate (PHB) production by these strains was determined by spectrophotometric method. Based on this quantitative estimation by u.v. spectrophotometer, 25 high PHB producing *Bacillus* strains were selected among the total 240 strains, which produced PHB ranging from 0.901g/l to 1.901 g/l. This ranged to a productivity of 50.05-70.04% PHB of cell dry weight (CDW). The correlation of PHB production with the cell dry weight was found to be statistically significant. The entire selected 25 *Bacillus* strains were classified to genus level by studying their morphological and biochemical characteristics.

**Key words:** *Bacillus* spp. STP. isolation, PHB, Identification, Soil.

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Polyhydroxyalkanoic acids (PHA) are common cellular granules found in prokaryotes (Anderson and Dawes 1990; Dawes and Senior 1973). Accumulation of these polymers under aerobic conditions often occurs when the carbon source is in excess but one or several other nutrients are limited (Anderson and Dawes 1990).

Polyhydroxyalkanoates (PHA) are receiving considerable attention because of their potential as renewable and biodegradable plastics and as a source of chiral synthons (Kessler *et al.*, 2001). Poly-3-hydroxybutyrate (PHB), a

representative compound of the family of PHA, has many potential applications in medicine, veterinary practice and agriculture due to its biodegradability and biocompatibility (Wang and Yu 2007). PHA have received increased attention because of their potential applications in areas of tissue engineering, environmental friendly packaging materials and as a chiral hydroxyalkanoate (HA) pool (Liu and Chen 2007).

Gram-positive bacteria, notably *Bacillus* and *Streptomyces*, have been used extensively in industry. However, these organisms have not yet been exploited for the production of the biodegradable polymers, PHA. Although, PHA have many potential applications, the cost of production has limited their use. Currently, medical applications is the only main area of use. Gram-negative bacteria, currently the only commercial source of PHA, have

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lipopolysaccharides (LPS) which co-purify with the PHA and cause immunogenic reactions. On the other hand, Gram-positive bacteria lack LPS, a positive feature which justifies intensive investigation into their production of PHA. More importantly, the Gram-positive genera *Corynebacterium*, *Nocardia* and *Rhodococcus* are the only wild-type bacteria, which naturally synthesize the commercially important copolymer, poly (3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV), from simple carbon source, such as, glucose. This will allow a considerable decrease in the cost of production of the copolymer (Valappil *et al.*, 2007). The genus *Bacillus* seems to be a potential candidate for the production of PHA, due to its better polymer yields and less stringent fermentation conditions. The novel PHA synthase discovered from *Bacillus* genus has ability to incorporate both scl and mcl PHA, indicating that the genus can be a potential producer of novel and known PHA with different ranges of monomeric compositions (Tajima *et al.*, 2003). Hence, the accumulation of PHB by the *Bacillus* genera has most distinct features which need further investigation.

Bacteria in the presence of rich nutrients tend to accumulate certain storage materials like: volutin granules, lipids and polyhydroxyalkanoates (Du *et al.*, 2004). Such nutrient rich ecosystems include: sewage sludge, polluted water and soil. Despite the presence of rich nutrients in these ecosystems, they have not been adequately explored for PHB accumulating bacteria in general and Gram positive *Bacillus* spp. in particular, hence, were considered to be the high potential environments for screening *Bacillus* spp. accumulating optimum PHB.

## MATERIAL AND METHODS

### Collection of samples

1 gram of each of 4 different soil samples were collected at the depth of 15 cm with a sterile spatula from the vicinities of Hussainsagar Lake, Hyderabad. Along with these samples, 5 sewage samples from different sites (inlet, intermediate, outlet, activated sludge and dry sludge) of Hussainsagar sewage treatment plant and the lake water sample were collected, during 2005. The samples collected were placed in sterile plastic

bags, bottles and stored at 4°C until further studies.

### Isolation of *Bacillus* spp.

A total of 10 samples (4 soil samples + 5 sewage samples + 1 lake water) were collected during the sampling period. Each of these samples (1g or 1ml) were suspended in 5 ml sterile distilled water and shaken vigorously for 2 min before heating them at 60°C for 60 min in a water bath. These heat treated liquid samples were serially diluted in sterile distilled water. The different dilutions ranging from 10<sup>-4</sup> to 10<sup>-8</sup> were plated on nutrient agar medium. Plates were then incubated at 30°C for 48 h.

### Cultivation

For the production of PHB, all of the 240 *Bacillus* spp. isolated from 10 various samples were cultivated in E2 mineral broth, a nitrogen limiting media (Lageveen *et al.*, 1988), with 2 % (w/v) glucose as carbon source. Each of these *Bacillus* isolates was grown in 250 ml Erlenmeyer flask containing 50 ml E2 mineral broth. After the inoculation with overnight grown 4% (v/v) inocula, the flasks were incubated at 30 °C for 48 h on an orbital shaker at 150 rev min<sup>-1</sup>.

### Extraction and quantitative assay of PHB

PHB was extracted from all of the 240 *Bacillus* isolates by using the hypochlorite method (Rawte and Mavinkurve 2002). Determination of the amount of PHB was performed chemically. Quantitative estimation of PHB extracted from the isolates was done by u.v. spectrophotometer method (Aslim *et al.*, 1998).

### Statistical analysis

The correlation between the production of PHB and cell dry weight of the isolates was determined by correlation-coefficient test (Wessa 2008).

$$\text{Correlation}(r) = \frac{N\Sigma XY - (\Sigma X)(\Sigma Y)}{\sqrt{([N\Sigma X^2 - (\Sigma X)^2][N\Sigma Y^2 - (\Sigma Y)^2])}}$$

where,

N = number of values or elements

X = first score

Y = second score

ΣXY = sum of the product of first and second scores

ΣX = sum of first scores

ΣY = sum of second scores

ΣX<sup>2</sup> = sum of square first scores

ΣY<sup>2</sup> = sum of square second scores

### Identification

Based on the UV spectrophotometer quantifications, 25 maximum PHB accumulating *Bacillus* isolates were selected from the total 240 *Bacillus* isolates as optimal PHB accumulating *Bacillus* spp. These 25 *Bacillus* spp. were tentatively identified based upon their morphological and biochemical characteristics. In the above process, *Bacillus* spp. were tested for Gram's reaction, spore morphology and the catalase test. The isolates were then characterized by their growth at various temperatures (5, 30, 45 and 65°C), tolerance at different salt levels (2, 4 and 10 g NaCl/100 ml) and reduction of nitrate. In addition, esculin, hippurate, casein and starch hydrolysis were examined. The production of acid from glucose, galactose, mannose, lactose, raffinose, xylose, cellobiose, fructose, salicin, mannitol, sucrose and maltose were tested. Along with urease, oxidase, and VP tests, the anaerobic growth of the *Bacillus* isolates was also

performed. The results obtained from these tests were compared with the standard taxonomic description from Priest and Alexander (Priest and Alexander 1988) and the *Bacillus* spp. were identified.

### Confirmation of the polymer monomers

Based on the UV spectrophotometer quantifications, 25 maximum PHB accumulating *Bacillus* isolates were selected amongst all the 240 *Bacillus* isolates. The amount of the polymer and the composition of the polymer in these selected 25 *Bacillus* isolates were confirmed using the advanced method, gas chromatography (Reddy *et al.*, 2008).

## RESULTS AND DISCUSSION

### Isolation of the *Bacillus* spp.

The *Bacillus* spp. were selectively isolated from the 10 different samples. For this, each of the 10 different samples (1g or 1ml) was

**Table 1.** PHB accumulated by the 25 selected *Bacillus* cultures

Isolates	<i>Bacillus</i> species	Dry wt(g/l)	PHB <sup>a</sup> (g/l)	% PHB(CDW)
87/I	<i>Bacillus</i> sp.	2.70	1.901	70.04
112/A	<i>Bacillus</i> sp.	2.70	1.827	67.63
99/G	<i>Bacillus subtilis</i>	2.70	1.765	65.37
79/A	<i>Bacillus megaterium</i>	2.18	1.289	59.12
76/G	<i>Bacillus sphaericus</i>	2.65	1.670	63.01
8/I	<i>Bacillus megaterium</i>	2.54	1.579	62.16
12/J	<i>Bacillus licheniformis</i>	2.42	1.465	60.53
25/C	<i>Bacillus sphaericus</i>	2.38	1.435	60.29
75/C	<i>Bacillus licheniformis</i>	2.15	1.248	58.04
69/I	<i>Bacillus flexus</i>	2.15	1.234	57.39
38/G	<i>Bacillus circulans</i>	2.04	1.123	55.04
18/C	<i>Bacillus circulans</i>	1.85	1.012	54.70
8/F	<i>Bacillus subtilis</i>	1.84	1.002	54.45
96/I	<i>Bacillus megaterium</i>	1.84	0.999	54.29
44/H	<i>Bacillus firmus</i>	1.85	0.998	53.94
97/B	<i>Bacillus subtilis</i>	1.85	0.992	53.62
49/B	<i>Bacillus cereus</i>	1.86	0.989	53.17
69/E	<i>Bacillus cereus</i>	1.85	0.980	52.97
29/C	<i>Bacillus flexus</i>	1.83	0.956	52.24
9/D	<i>Bacillus firmus</i>	1.84	0.967	52.55
89/C	<i>Bacillus megaterium</i>	1.82	0.950	52.19
20/F	<i>Bacillus circulans</i>	1.80	0.923	51.27
84/F	<i>Bacillus megaterium</i>	1.80	0.923	51.27
10/E	<i>Bacillus subtilis</i>	1.80	0.912	50.66
49/C	<i>Bacillus flexus</i>	1.80	0.901	50.05

Key: CDW =cell dry weight, a = amount of PHB determined by GC.

first suspended in 5 ml sterile distilled water in a test tube and was heated at 60°C for 60 min in a water bath. In such high temperatures only spore forming bacteria would survive, which were later isolated by plating onto nutrient agar medium. A total of 240 *Bacillus* spp. were isolated from the 10 different samples studied during 2005.

#### Quantitative estimation of the PHB

Each of the 240 *Bacillus* spp. was first grown in 50 ml E2 media broth in 250 ml flasks and was employed to extract PHB after two days of incubation on an orbital shaker as described previously. The PHB from the isolates was extracted by the hypochlorite method (Rawte and Mavinkurve 2002). A wide variety of bacteria are known to accumulate PHB, these bacteria have been reported from various environments. The amounts of PHB extracted from 337 bacterial isolates screened from the tropical marine ecosystem accumulated as high as 1.73g/l PHB in nitrogen limiting E2 medium (Rawte *et al.*, 2002). *Bacillus* spp. isolated from soil have been reported to accumulate 0.004 - 0.097 g/l PHB when grown in nutrient broth (Yilmaz *et al.*, 2005), while *Methylobacterium* sp. isolated from a pond is reported to accumulate 1.1g/l PHB utilizing cheese whey (Yellore and Desai 1998). In a recent report, 15 PHB accumulating bacterial isolates (of which, 7 were *Bacillus* spp.) from municipal sewage sludge accumulated 0.78 – 1.65 g/l PHB when grown in E2 medium with 2% glucose (Reddy *et al.*, 2008).

During this study, the polymer accumulation of 240 *Bacillus* isolates, isolated from 10 different samples was quantified by UV spectrophotometer method (Aslim *et al.*, 1998). The amount of PHB formed from these 240

*Bacillus* isolates varied considerably. Some of these isolates accumulated negligible amount of PHB. Twenty five *Bacillus* spp. were selected after the quantification of all the 240 *Bacillus* isolates, which accumulated PHB in the range of 0.901g/l to 1.901 g/l. These were selected as the optimal PHB accumulating *Bacillus* isolates. All these results were tabulated in Table 1. These 25 selected *Bacillus* isolates were grown in E2 broth to estimate cell wet weight, cell dry weight (CDW), amount of PHB produced and percentage of PHB/cell dry weight.

The yield of PHB accumulated by these 25 *Bacillus* isolates amounted from 50.05-70.04% of their CDW (Table 1) signifying the potentials of the optimal PHB accumulating *Bacillus* spp. in these ecosystems.

It was investigated whether any relationship existed between the cell dry weight and PHB production and the correlation was found  $r = 0.97$ . Since 0.97 is very near to +1.0, there is a strong correlation between the two variables. Only a few reports focused on the potential of utilizing resident *Bacillus* species from activated sludge in polyhydroxybutyrate production, as well as, the production of PHB from food wastes. They have attractive properties, such as, short generation time, absence of endotoxins, and secretion of both amylases and proteinases that can well utilize food wastes as nutrients, which can further reduce the cost of production of polyhydroxyalkanoates (Law *et al.*, 2001).

#### Identification of *Bacillus* spp

The selected 25 *Bacillus* spp. were further studied for their morphological and biochemical characteristics. Based on Priest and Alexander (Priest and Alexander 1988), all these

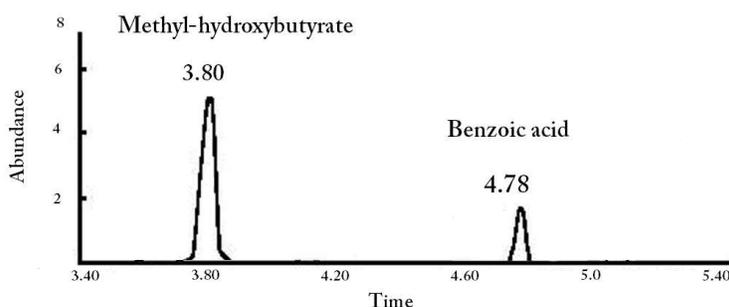


Fig. 1. GC chromatogram of PHB

selected 25 *Bacillus* spp. were tentatively classified using their morphological and biochemical characteristics.

#### Confirmation of the polymer monomers

The extracted polymers from the high PHB producing 25 *Bacillus* isolates were confirmed as PHB homopolymers by GC (Fig. 1).

#### CONCLUSIONS

In the present study, the selection of 25 *Bacillus* spp. out of 240 *Bacillus* spp. was based on the highest amount of PHB produced by these isolates after growth with 2% glucose as sole carbon source. These 25 *Bacillus* spp. produced PHB from 0.901 g/l to 1.901g/l amounting to about 50.05-70.04% PHB of cell dry weight (Table 1), i.e. 70.04% was the highest report of PHB accumulating *Bacillus* sp. when grown on the media supplemented with 2% glucose, whereas, Rohini et al., (2006) reported 64.10% PHB of CDW from soil bacteria when grown on the media supplemented with glycerol.

The further study involves the production of PHB by the selected *Bacillus* spp. with cheap carbon substrates and the characterization of the polymers produced.

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