# Production of poly-(3-hydroxybutyrate) by Native *Bacillus* Isolates from Agricultural Wastes

## M. Thirumala*, S. Vishnuvardhan Reddy and S.K. Mahmood

Environmental Microbiology Lab, Department of Botany,
Osmania University, Hyderabad - 500 007, India.

**Two native *Bacillus* isolates produced PHB using agricultural wastes like wheat straw, rice straw, sugarcane bassage, corn cobs and saw dust. *Bacillus* sp. 87I produced 50.9% (1.5 g/l PHB), when sugars from sugarcane bassage were used in E2 media. The second *Bacillus* sp. 112A produced 47.5 % (1.1 g/l) of PHB, when filtrate from sugarcane bassage fermentation was used as carbon substrate. The native *Bacillus* spp. 112A and 87I have desirable properties of tolerance to high conditions of pH and temperatures.**

**Key words:** Agricultural wastes, *Bacillus* sp., Isolates, PHB, Production.

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

PHAs are receiving considerable attention because of their potential as renewable and biodegradable plastics and as a source of chiral synthons (Kessler *et al.,* 2001). Polyhydroxybutyrate (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 applications in areas of tissue engineering, environmental friendly packaging materials and as a chiral hydroxyalkanoate (HA) pool (Liu and Chen, 2007).

It has been studied that the cost of carbon source is critical for reducing the production cost of PHB (Akiyama *et al.,* 2003; Choi and Lee, 1997; Choi *et al.,* 1999). This high production cost of PHB can be decreased by strain development, improving fermentation process, separation process and by using a cheap carbon source (Kim, 2000). In PHB production, about

* To whom all correspondence should be addressed.
E-mail: vishnu_micro@rediffmail.com

50% of the total production cost is for raw material (Anderson and Dawes, 1990).  Thus, the use of an alternate cheap carbon source is required in order to reduce the high production cost of PHB. In this paper, the abundant, cheap and renewable carbon sources, such as, wheat straw, rice straw, sugar cane bassage, corn cobs and saw dust were studied for PHB production. These raw materials were tested with the two isolates (*Bacillus* sp. 87I and *Bacillus* sp. 112A).

## MATERIAL AND METHODS

### Isolation and identification of two native *Bacillus* isolates

Two *Bacillus* spp. were isolated from activated sludge samples and identified by their physiological and morphological characteristics. Production of PHB from agricultural wastes

### Raw materials

Wheat straw, rice straw, sugarcane bassage, corn cobs and saw dust were used as cellulose containing raw materials for the production of PHB. Each raw material was dried at $50°\pm2°C$, ground to powder form and then sieved through a 1mm sieve. Powder of each raw material was initially treated for producing reducing sugars. These sugars were later used as carbon substrates for the production of PHB.

### Microorganisms

*Aspergillus niger* and *Trichoderma reesei,* both cultures obtained from Mycology and Plant Pathology Lab, collection centre of Department of Botany, Osmania University were used for the production of cellulase enzymes and thereafter the reducing sugars from the raw materials. These cultures were preserved on potato dextrose agar (PDA) slants.

### Inoculum preparation

Fungal cultures were inoculated on PDA medium in the Petri plate. After 4-5 days of growth, these cultures in the form of culture discs were used for inoculation.

### Pretreatment

Each raw material was pretreated with 0.5 M sodium hydroxide at 1:10 (solid: liquid) ratio, for 1 h at 15 psi and the residue was freed of alkali by washing with water and dried at $50 \pm 2°C$ for subsequent use.

### Culture medium

The Mandle's medium (Bollok and Reczey, 2000) used here contained (g/l): Urea- 0.3,  $(NH_4)_2SO_4$ - 1.4, $KH_2PO_4$ - 2, $CaCl_2$ - 0.3, $MgSO_4 7H_2O$ - 0.3, Peptone -  0.75, Yeast extract - 0.25 and Trace elements - 1% (v/v) [Composition (ml/l):$FeSO_4 7H_2O$ - 0.5, $MnSO_4$ - 0.16, $ZnSO_4$ - 0.14, $CoCl_2$ - 2.], pH was adjusted to 5.5 - 6.0 before sterilization.

### Fungal treatment

10g of each pretreated raw material was taken in a conical flask containing 200ml of Mandle's medium. The conical flasks were plugged with cotton and sterilized at 15 lbs for 20 minutes. Each flask was inoculated with 4-5 discs of fungal cultures. These flasks were incubated at room temperature for 5days on an orbital shaker. After five days, the mycelium was separated by filtration through Whatman filter paper No.1. The filtrate was then used for estimation of total sugars and for further studies.

### Determination of total sugar content

The carbohydrate content of untreated and treated raw materials in the culture filtrate was measured by phenol sulphuric acid method (Dubois *et al.,* 1956) using standard graph.

### Production of PHB

The above obtained filtrate, containing the free reducing sugars was used as a substitute for carbon substrate in the E2 medium (Lageveen *et al.* 1988) for PHB production. In this sugar rich culture filtrate, all the other ingredients of E2 media were added and autoclaved at 110°C for 10 min. The two isolates, *Bacillus* sp. 87I and *Bacillus* sp. 112A were then grown in this medium in 250 ml flasks for 2 days with incubation at 28°C on a rotatory shaker at 150 rpm.

### Extraction of PHB from the isolate

After the incubation, PHB was extracted from the two isolates (*Bacillus* sp. 87I and *Bacillus* sp. 112A) by using the Hypochlorite method (Rawte and Mavinkurve, 2002).  Cell suspension was centrifuged at 6000 rpm for 10 min. The cell pellet was washed once with saline and was recentrifuged to get the pellet. The cell pellet was then suspended in sodium hypochlorite and incubated at 37°C for 10 min with stirring. This extract was centrifuged at 8000 rpm for 20 min and the pellet of PHB was washed with cold

diethyl ether. The pellet was again centrifuged at 8000 rpm to get purified PHB.

**Analytical methods**

After incubation, the following analysis was done. Each sample was used for the determination of the cell dry weight (CDW) and PHB content in the culture fluid. The cell concentration was determined by measuring CDW: 5 ml culture broth was centrifuged, pellet obtained was washed and dried at 105°C until the weight did not decrease further. The PHB extracted from the two isolates (*Bacillus* sp. 87I and *Bacillus* sp. 112A) by the above method were quantified by UV spectrophotometer method (Yilmaz et al. 2005; Aslim et al. 1998). PHB (%) was defined as the percentage of the ratio of PHB to CDW.

## RESULTS AND DISCUSSION

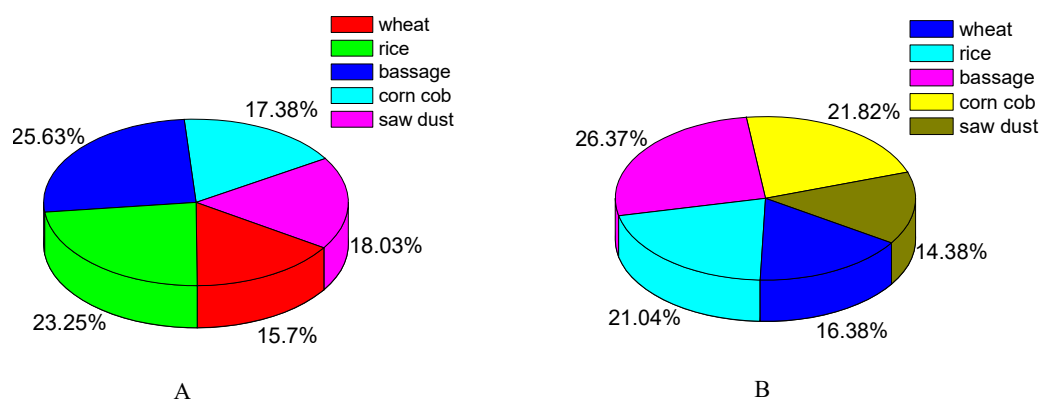**Production of PHB from agricultural wastes**

Many workers have explored various easily available agro-industrial wastes as carbon sources, such as, sugarcane molasses, date syrup, soy molasses for the production of PHA employing *Bacillus* (Gouda *et al.*, 2001; Omar *et al.,* 2001; Full *et al.,* 2006).

The raw materials used in the present study contained cellulose in the range of 45-35% apart from hemicelluloses from 25-10%, lignin from 10-6%, total nitrogen from 3-1.0% and ash contents from 20-15%. As observed in a number of previous studies, the size of the raw material is very much important for heat transfer and enzymatic hydrolysis. Hence, >1mm size of these raw materials were selected by sieving. The pretreatment of the raw material with sodium hydroxide was meant for delignification.

After pretreatment, each of the raw materials was added to Mandle's medium at 10 g/200 ml concentration. To observe the efficiency of alkali pretreatment to raw materials, samples without any alkali pretreatment were also used for fermentation experiments. Alkali pretreatment was not prerequisite for the growth of the fungi, but, as documented and proved in a number of studies, it could increase the amount of reducing sugars produced by a factor of 5. This can be explained as, delignification with alkali treatment makes the lignin component to break into simple compounds, which would latter be degraded by the fungal cultures into further simpler reducing sugars. Hence, the present study involved alkali pretreatment of the raw materials. Total sugars, reducing sugars, non-reducing sugars, organic carbon, nitrogen, total solids and moisture content of each of the raw materials were determined. The initial composition of the raw materials used in this study was shown in Table 1. In the sugarcane bassage the amount of total sugars, moisture content, non reducing sugars and organic carbon were highest, when compared to the other four raw materials used.

All the five raw materials were subjected to grinding and sieving through 1mm sieve and pretreated with alkali as mentioned above. After



**Fig. 1.** Pie diagrams of preferential level of agricultural wastes for PHB production by *Bacillus* sp. 87/I (A) and *Bacillus* sp. 112/A (B)

alkali pretreatment, each raw material was mixed with the Mendle's medium at 10g/200ml concentration and was sterilized by autoclaving at 121°C for 20 min. Finally, the flasks were inoculated with the inoculums already prepared separately. After incubation of five days, the medium was filtered to remove the mycelium and the culture filterate was used to estimate the amount of total sugars by phenol sulphuric acid method (Table 2).

Autoclaving for sterilization has affected and resulted in increase in sugar content. With fungal treatment still increase in the yield of sugars was observed. The combination of two fungi, *Aspergillus niger* and *Trichoderma reesei,* resulted in high yield of sugars than individual fungal treatment with all raw materials except rice straw, in which this combination was not effective. Rice straw produced 29 mg/g total sugar content, when treated only with *T. reesei.* In wheat straw,

the use of both *A. niger* and *T. reesei* was more effective, when compared to other raw materials. On the other hand, the highest amount of total sugars was found in medium containing sugarcane bassage as the raw material with dual fungal treatment. Individually, *T. reesei* treatment was more effective in increasing the total sugar content when compared to *A. niger,* in all the raw materials used (Table 2).

A modified E2 media was used for the production of PHB by the two isolates, *Bacillus* sp. 87I and *Bacillus* sp. 112A, from these agricultural waste raw materials. The carbon substrate in the E2 media was substituted by the reducing sugars produced by the fungal treatments. The high sugar yields of different raw materials from fungal treatments were used for the production of PHB by *Bacillus* sp. 87I and *Bacillus* sp.112A.

**Table 1.** The initial composition of various raw materials

| Raw materials | Total sugars (mg/g) | Reducing sugars (mg/g) | Non-reducing sugars (mg/g) | Moisture (%) | Total solids (%) | Organic carbon (%) | Nitrogen (%) |
|---|---|---|---|---|---|---|---|
| Wheat straw | 0.5 | 0.0115 | 0.4885 | 5.324 | 95.5 | 36.2 | 0.15 |
| Rice straw | 0.6 | 0.0134 | 0.5866 | 1.945 | 97.8 | 36.4 | 0.45 |
| Sugarcane bassage | 1.5 | 0.0147 | 1.4853 | 8.543 | 91.6 | 37.2 | 0.46 |
| Corn cobs | 0.8 | 0.0180 | 0.7810 | 3.456 | 90.3 | 36.8 | 0.50 |
| Saw dust | 0.4 | 0.0175 | 0.3825 | 2.345 | 87.9 | 29.5 | 0.21 |

**Table 2.** Effect of fungal treatments on the amount of total sugars (mg/g) in different raw materials

| | Wheat straw | Rice straw | Sugarcane bassage | Corn cobs | Saw dust |
|---|---|---|---|---|---|
| Untreated (only autoclaving) | 15 | 25 | 32 | 17 | 12 |
| *A. niger* | 17 | 21 | 35 | 19 | 15 |
| *T. reesei* | 18 | 29 | 38 | 21 | 17 |
| *A. niger + T. reesei* | 35 | 25 | 39 | 24 | 18 |

**Table 3.** PHB (g/l) and PHB (%) produced from agriculture wastes by *Bacillus* sp. 87I and *Bacillus* sp. 112A

| | Wheat straw | Rice straw | Sugarcane bassage | Corn cobs | Saw dust |
|---|---|---|---|---|---|
| 87I | 0.9 (31.0 %) | 1.1 (45.9%) | 1.5 (50.9 %) | 0.9 (34.3%) | 0.7(35.6%) |
| 112A | 0.6 (29.5%) | 0.8 (37.9%) | 1.1 (47.5%) | 0.8 (39.3%) | 0.9 (25.9%) |

The amount of PHB and the PHB % per CDW produced by *Bacillus* sp. 87I and *Bacillus* sp.112A, when grown on modified E2 media from different raw materials were shown in Table 3. The *Bacillus* sp. 87I produced 50.9% (1.5 g/l PHB), when sugars from sugarcane bassage were used in E2 media. This was followed by sugars from rice straw and saw dust. Whereas, when the sugars of raw materials, wheat straw and corn cobs were used, they produced 0.9 g/l PHB. Contrary to this was the PHB % per CDW, which was 31.0% in case of wheat straw filtrate and 34.3% for corn cob filtrate because of the difference in the amount of biomass and cell dry weight produced when these two raw material filtrates were used separately as sole carbon source. The second *Bacillus* sp. 112A also produced the high amount (1.1 g/l) of PHB, when filtrate from sugarcane bassage fermentation was used as carbon substrate. Wheat straw filtrate produced least amount (0.6 g/l) of PHB. Saw dust filtrate was more effectively utilized when compared to the first isolate and resulted in production of 0.9 g/l PHB. Rice straw and corn cobs filtrates showed similar results (0.8 g/l PHB). Though, *Bacillus* sp. 112A showed similar values of PHB g/l with rice straw and corn cobs filterate, PHB % was different, when these two culture filtrates were separately used as carbon source. The highest value of PHB% per CDW of *Bacillus* sp. 112A was 47.5% and the least was 25.9%. The pie diagrams of the preferential levels of these agricultural wastes for each of these two *Bacillus* isolates (*Bacillus* sp. 87I and *Bacillus* sp. 112A) were presented in Fig. 1.

These diagrams showed that, sugar cane bassage were the raw material of choice for PHB production by both the isolates. This was followed by rice straw, saw dust, corn cobs and wheat straw in case of *Bacillus* sp. 87I. On the other hand, sugarcane bassage was followed by corn cobs, rice straw, wheat straw and saw dust for *Bacillus* sp. 112A. Though, the two *Bacillus* isolates showed difference in utilization of sugarcane bassage and in the amount of PHB production when it was used as a raw material, it was the material which yielded the highest amount of polymer by both the *Bacillus* isolates when compared to all the raw materials studied. Hence, we can conclude that the sugarcane bassage is an important raw material for the production of PHB by *Bacillus* spp.

The native *Bacillus* spp. 112A and 87I have desirable properties of tolerance to high conditions of pH and temperatures. Such strains are found to be better suited for industrial production of PHB in order to minimize contamination (Tajima *et al.,* 2003). Not only this, the nonpathogenic features ensure safety while handling and the ability to ferment various carbohydrates by recycling agro-industrial wastes can reduce cost of production and environmental pollution (Yu, 2001).

## ACKNOWLEDGMENTS

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