

## **Polyhydroxybutyrate Production using Agro-industrial Residue as Substrate by *Bacillus sphaericus* NCIM 5149**

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### **ABSTRACT**

*The aim of this work was to study the production of polyhydroxybutyrate (PHB) using agro- industrial residues as the carbon source. Seven substrates, viz., wheat bran, potato starch, sesame oil cake, groundnut oil cake, cassava powder, jackfruit seed powder and corn flour were hydrolyzed using commercial enzymes and the hydrolyzates assessed for selecting the best substrate for PHB production. Jackfruit seed powder gave the maximum production of PHB under submerged fermentation using *Bacillus sphaericus* (19%) at the initial pH of 7.5.*

**Key words:** *Bacillus sphaericus*, Polyhydroxybutyrate, Jackfruit seed powder

### **INTRODUCTION**

The history of plastic begins from 1862 by Alexander Parkes. The superior characters such as durability, strengths, shape and moldable oblige mankind incredibly depend on plastic for their daily life. According to the statistics in 2000, the annual consumption of plastics in India was about to reach 4kg/person/year, and that of world average of 24.5 kg. By 2010, global consumption of plastics is expected to reach up 258 mt/year from 180mt/year (Association of German Plastics Manufacturers, 2001). Since these plastics have high molecular weight and tightly bonded together, these are not degradable, which makes their disposal difficult and inversely leads to negative impact on the environment. Thus, the concept of biodegradable plastic came as a solution for this problem, which could be degraded

by the microorganism in the environment when proper conditions such as the sunlight, moisture, oxygen, etc are available (Abe and Doi, 2002). The degradation rate of P(3HB) ranges in the order of a few months (in anaerobic sewage) to years (in seawater) (Jendrossek et al., 1996; Mergaert et al. 1994; Mergaert et al., 1993). The key factor for their high cost is due to the narrow production to achieve the large economies of the scale. The cost of carbon source, fermentation process and the downstream processing of the polymer contribute to the high cost of manufacturing process of the polymer (Choi and Lee, 1999). About 50% of the production costs of the polyhydroxyalkanoates (PHA) are added by the cost of carbon source (Halami, 2008). PHA are biodegradable, water insoluble, non-toxic, bio-compatible, piezoelectronic, thermoplastic, and/or elastomeric. These features make them suitable for applications

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in the packaging industry and as substitute for hydrocarbon-based plastics (Anderson and Dawes, 1990). It has wide applications in different areas such as packaging material, long term dosage of drugs, medicines, insecticides, herbicides, fertilizers cosmetic world, disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetics containers, shampoo bottles, cups etc. Studies are progressing for its relevance in medical field for bone replacements and plates, surgical pins, sutures, wound dressings, and blood vessel replacements.

Polyhydroxybutyrate is the intracellular granule, synthesized by bacteria and acts as an energy storage facility. In some *Bacillus* sp, it supplies energy for sporulation (Slepecky and Law, 1961). The low molecular weight P(3HB) is a part of bacterial  $\text{Ca}^{2+}$  channels (Lara et al., 1999). The storage granules are synthesized by the microorganisms when the cell's surroundings contain an unbalanced growth condition such as limited concentration of O, N, P, S, or trace elements, e.g., Mg, Ca, Fe and high carbon concentration (Lee, 1996, Sudesh et al., 2000; Kessler and Witholt, 2001). Under normal growth conditions, the nutrient sources are used for the synthesis of proteins essential for the growth in bacteria. The nitrogen source depletion leads to the cessation of protein synthesis, which in turn leads to the inhibition of TCA cycle enzymes such as citrate synthase and isocitrate dehydrogenase and consequently slows down the TCA cycle. As a result, the acetyl-CoA routes to P(3HB) biosynthesis (Dawes and Senior, 1973). Both the shortening of external nutrients and internal sources such as RNA or enzymes facilitate the PHA synthesis. The aim of this work was to study the production of polyhydroxybutyrate (PHB) using agro- industrial residues as the carbon source.

## MATERIALS AND METHODS

### Microorganism and inoculum preparation

*Bacillus sphaericus* 5149 was obtained from the NCIM (Pune) and maintained on agar slants and Petri dishes containing Luria Bertani agar media. The primary inoculum was prepared in Luria Bertani medium in 250 ml Erlenmeyer flask containing 50 ml of sterile medium (autoclaved at 121.5°C for 15 minutes) and inoculated from the stock culture. After 16 h incubation at 30°C and

200 rpm, 2 ml of culture was taken to inoculate the flask containing 100 ml of sterile cultivation medium, which contained (g/L):  $(\text{NH}_4)_2\text{SO}_4$ , 2;  $\text{KH}_2\text{PO}_4$ , 2;  $\text{Na}_2\text{HPO}_4$ , 0.6;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2; Yeast extract, 0.2; Fructose, 10; and 0.1 ml trace element solution. The trace element solution contained (g/L):  $\text{H}_3\text{BO}_3$ , 0.01;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.02;  $\text{CuSO}_4$ , 0.01;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1;  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ , 0.02. Fructose and trace element solution were autoclaved separately and reconstituted prior to the inoculation. The flasks were incubated at 30°C and 200 rpm for the requested time.

### Growth kinetic studies of the culture

The above described medium containing fructose as sole carbon source was used for the kinetic studies. Fermentation was carried out as above for 36 h. At regular intervals, the samples were removed (as whole flasks in duplicate) and analyzed the biomass, PHB production, reducing sugars and protein.

### Effect of inoculum size and age

Different inoculum size varying from 1 to 5 ml was tested for the effect of inoculum size on PHB production. Also, the optimization of pre-inoculum age from 12 to 24 h with an interval of 4 h was carried out. To find out the number of the cells in the inoculum size, serial dilution of the inoculum was done and plated on Luria-Bertani agar media and the colonies were counted after incubated at 30°C for 24 h.

### Effect of pH on PHB production

Different initial pH of the medium (5.0 to 8.0) was used to check whether pH has any noticeable effect on PHB production. The initial pH of the medium was adjusted by 1N hydrochloric acid or sodium hydroxide.

### Evaluation of agro-industrial residues as substrate

Seven agro-industrial residues, viz., wheat bran, potato starch, sesame oil cake, groundnut oil cake, cassava powder, jackfruit seed powder and corn flour were assessed for selecting the best substrate for PHB production. These were gelatinized, liquefied and saccharified as described by John et al. (2006). The conditions were: gelatinization at 100°C for 15 min, followed by liquefaction with alpha amylase (Novo Termamyl, 5000 IU/ml) at 85°C, pH 5 for 30 min and then saccharification

with glucoamylase (Novo AMG, 2000 IU/ml) at 60<sup>o</sup> C for 70 min. The hydrolyzate obtained was filtered through a muslin cloth and the clear hydrolyzate containing reducing sugar was used as the sole carbon source for the PHB production. The reducing sugar in the hydrolyzate was measured using dinitrosalicylic acid (DNS) method (Miller, 1959).

### Determination of PHB

**Alkaline digestion:** The pellet was collected by centrifugation at 8000 x g for 20 min and lyophilized. The lyophilized pellet was digested with 30% sodium hypochlorite solution at 37<sup>o</sup>C for 20 min. The spectrophotometric assay was done as described by Law and Slepecky (1960). The residue was collected by centrifugation at 8000 x g for 20 min and performed a series of washing steps using water, acetone and finally ethanol. The polymer was dissolved in chloroform and kept for complete evaporation. Then 5ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added and heated for 40 min at 100<sup>o</sup> C in a water bath. The resultant crotonic acid was measured at 235 nm against H<sub>2</sub>SO<sub>4</sub> as blank in a spectrophotometer (Shimadzu 361A, Japan).

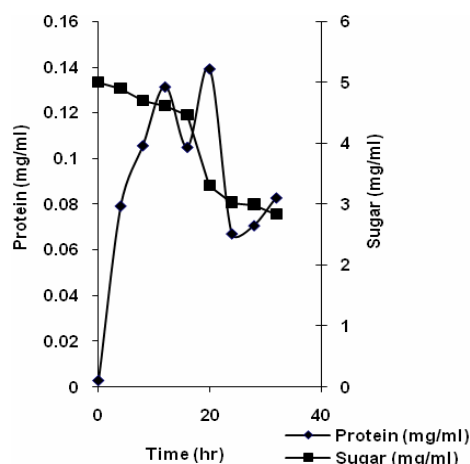
## RESULTS AND DISCUSSION

### Growth kinetic studies of the culture

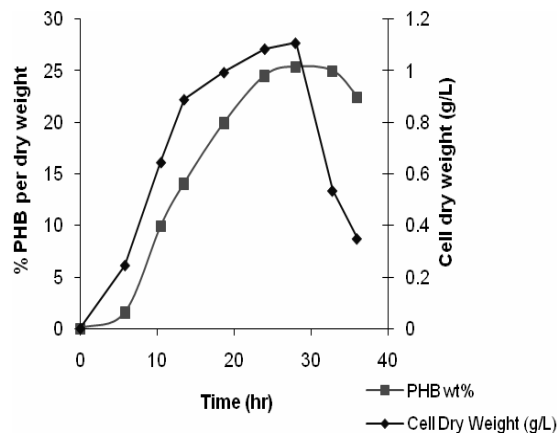
The kinetic study of growth of the culture was carried out to at pH 7 and the concentration of fructose was 5mg/mL. The reduction in the reducing sugar was an indicator for the ability of

the microorganism to consume fructose as the carbon source. After 16<sup>th</sup> h, there was a sharp decrease in the concentration of fructose probably due to the starting of log phase where cellular metabolism was in its peak (Fig 1). The protein concentration in the medium was estimated using Lowrey's method (Lowry et al., 1951), which did not show any organized format. At the initial hour of incubation, the protein concentration was very low, after that the concentration increased. As the incubation time progressed, various enzymes might be synthesized in order to facilitate the cell growth and for other metabolic needs. After 16<sup>th</sup> h, protein concentration increased and at the same time, there was a decrease in the fructose concentration in the medium. This confirmed that this was the phase in which active metabolism was taking place. From 24<sup>th</sup> h again the protein profile increased, possibly due to the start of sporulation in the bacterium.

Fig. 2 shows the biomass and PHB production at particular intervals. The PHB yield and biomass concentration increased until 28<sup>th</sup>h of fermentation and were 25% and 1.1g/L, respectively. The rapid drop in cell dry weight (CDW) was due to the cell growth decline phase. The slight decrease in PHB production could be due to the fact that the microorganism could synthesize PHB until the sporulation stage and after that the remaining bacterial cells consume the PHB (Benoit, 1990; Nam and Ryu, 1985). Yilmaz et al., (2005) reported that some *B. sphaericus* strains were able to synthesize PHB up to 32.55% (w/v).



**Figure 1** - Residual sugar and protein in the fermentation medium.



**Figure 2** - Content of PHB and dry weight of the *B. sphaericus* as a function of incubation time

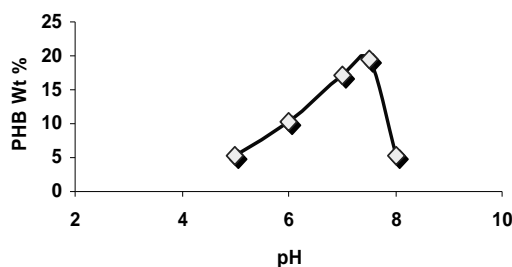
### Effect of inoculum age and size

Pre-inoculum of 16h age gave maximum PHB production. The pre-inoculum prepared in the Luria-Bertani medium was rich in nitrogen concentration could involve in protein synthesis in the bacterium and thus increased the biomass. When these cells were put in the production medium, easy assimilable carbon source facilitated the growth of microorganism. Maximum production was obtained with 2 ml of preinoculum of 16 h age, which gave about 25% PHB (data not shown). The inoculum size of the culture

determined was  $8 \times 10^8$  CFU/mL.

### Effect of pH on PHB production

Change in initial pH of the medium showed a strong influence on the production of PHB. Even a slight difference in pH from the optimum point denoted a sudden reduction in PHB production. As shown in the Fig. 3, medium with initial pH value of 7.5, gave the maximum production of PHB of 25%. The drastic change in production seems to be due to the effect of initial pH on the bioavailability of trace elements.



**Figure 3** - PHB production by *B. sphaericus* at different initial pH

### Evaluation of agro-industrial residues as carbon substrate

The major restriction in the commercialization of bioplastic is their high production cost. The use of readily available cheap agro-industrial residues as the carbon sources may reduce the higher cost. Several studies have shown the utilization of

various carbon sources for different bacterial strains. The glycerol utilization as a carbon source was reported by Taidi et al. for *Ralstonia. eutroph* (Taidi et al., 1994). The culture showed no difference in the PHB production in media containing sucrose and glycerol as carbon source. Fatty acids from the fermented fruit and vegetable

residues also can be supplemented as a carbon source for the microorganisms, to reduce the production cost (Ganzeveld et al., 1999; Nonato et al., 2001). There are reports describing 25g/L of PHB when soluble starch was utilized as a carbon source with *Azetobacter chroococcum* in fed-batch mode (Kim, 2007). PHB production by 11 different *Bacillus* sp. was studied by Chen et al. (1991). Results showed a maximum of 50% (w/v) PHB of dry cell weight of the bacteria. In one study, 29 *Bacillus* strains were assessed for PHB production and found that *B. megaterium* showed maximum production of 0.207g/L and productivity percentage of 48.13%. Lowest PHB was 6.53% in *B. subtilis* K1 (Aslim et al., 2002).

Table 1 shows the PHB production in the hydrolyzates of different agro-industrial residues. Van-Thuoc et al., (2007) reported rgar in order to use the agro-industrial residues as fermentation substrates, these should be subjected to hydrolysis step for the release of easily metabolizable sugars, Acid and enzymatic methods are the two main reported methods for hydrolysis. But acid hydrolysis requires more energy for heating and is relatively difficult to control. It also necessitates corrosion resistant materials since it gives rise to high color and salt and ash content. The enzymatic hydrolysis of sesame oil cake and groundnut oil cake generate only low quantity of reducing sugar and that may be the reason why PHB production became inferior. The enzymatic hydrolysis of sesame oil cake and groundnut oil cake generated only low quantity of reducing sugar that could be the reason why PHB production was inferior from these. Reducing sugar from wheat bran was good and the biomass production was appreciable, but this also showed poor yield of PHB. In a study,

Van-Thuoc et al., (2007), using wheat bran hydrolyzate as the carbon source, reported the PHB concentration as 1.08g/L, which was almost similar to the result of this study (1.06 g/L) but the PHB content was 6.8% in this study in comparison to the reported value of 33.8% by Van-Thuoc et al., (2007). The wheat bran hydrolysate is rich in protein concentration (Van-Thuoc et al., 2007). Although the production medium contained the yeast extract (0.2g/L), the protein in the hydrolyzate might have contributed to the cell growth (CDW 15.5 g/L). The cassava bagasse hydrolyzate also resulted good growth (CDW 2.5g/L) but the amount of PHB was low (Table 1). Several agro-industrial residues such as potato starch, babassu, soy cake (Oliveira et al., 2004) cane molasses, whey (Ahn et al., 2001) have been reported for PHB production. Fukui and Doi (1998) reported that the plant oils such as olive oil, corn oil and palm were good carbon substrates for *R. eutropha* for PHB production. Thakor et al. (2005) found the coconut oil as one of the best carbon source for *Comamonas testosteroni*. Rusendi and John (1995) used waste potato starch hydrolyzate for the production of PHB and reported a yield of 77% of the biomass dry weight. In the present study, there was only slight difference in PHB production in potato starch and jackfruit seed powder. , However, jackfruit seed powder was selected as the substrate for further studies due to its relative cheaper cost. The reducing sugar obtained by the enzymatic hydrolysis of jackfruit seed powder was 0.1g/mL (data not shown). Bobbio et al. (1978) reported that jackfruit contained 31.9% protein, 1.3% crude lipids and 66.2% carbohydrates on dry weight basis.

**Table 1** - Comparison of synthesis of PHB on hydrolyzates prepared from different agro-industrial residues (2%)

Carbon Substrate	CDW (g/L)	PHB Concentration (g/L) <sup>a</sup>	PHB content (%) <sup>b</sup>
Corn flour	1.5	0.049	3.3
Wheat bran	15.5	1.065	6.8
Cassava bagasse	2.5	0.161	6.4
Jackfruit seed powder	1.5	0.690	46.0
Potato starch	1.5	0.710	47.0
SOC	1.0	0.146	14.6
GOC	1.5	0.280	18.7

<sup>a</sup> Gram PHB per liter of culture

<sup>b</sup> Percentage of PHB in cell dry weight

## CONCLUSION

From the results it was concluded that the enzyme hydrolyzate of jackfruit seed powder could be used as a good substrate for PHB productions. Maximum production was obtained when a hydrolyzate prepared from 2% jackfruit seed powder was fermented for 36 h at 30°C in a medium containing nutrient salts and initial pH as 7.5. The PHB content in the cells was 46%.

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## REFERENCES

- Abe, H.; Doi, Y. (2002), Molecular and material design of biodegradable poly(hydroxyl-alkanoate)s', in Doi Y and Steinbuchel A, *Biopolymers 3b, Polyesters II*, Weinheim, Wiley-VCH, 105-132.
- Anderson, A.J. and Dawes, E. A. (1990), Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol Rev.*, **54**, 450-472.
- Ahn W. S.; Park, S. J and Lee, S. Y. (2001), Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant *Escherichia coli*. *Biotechnol. Lett.*, **23**, 235-240.
- Aslim, B.; Yuksekdog Z. N. and Beyatli Y. (2002), Determination of growth quantities of certain *Bacillus* Species isolated from soil. Turkish electronic journal of biotechnology, special issue, 24-30.
- Association of German Plastics Manufacturers (2001), Plastics: Business Data and Charts, VKE Working Group Statistics and Market Research.
- Benoit, T. G.; Wilson, G. R.; Baygh, C. L. (1990), Fermentation during growth and sporulation of *Bacillus thuringiensis* HD-1. *Lett. Appl. Microbiol.* **10**, 15-18.
- Bobbio, F. O.; El-Dash A. A.; Bobbio, P. A. and Rodrigues, L. R. (1978), Isolation and characterization of the physicochemical properties of the starch of jackfruit seeds (*Artocarpus heterorphyllus*). *Cereal Chem* **55**, 505-511.
- Chen, G.Q.; Ko' nig, K. H. and Lafferty, R. M. (1991), Occurrence of poly-D(-)-3- hydroxyalkanoates in the genus *Bacillus*. *FEMS Microbiol. Lett.*, **84**, 174-176.
- Choi, J. and Lee, S. Y. (1999), Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation *Appl Microbiol Biotechnol.*, **51**, 13-21.
- Dawes, E. A. and Senior, P. J. (1973), The role and regulation of energy reserve polymers in microorganisms. *Adv. Microbiol. Physiol.* **10**, 135-266.
- Fukui, T. and Doi, Y. (1998), Efficient production of polyhydroxyalkanoates from plant oils by *Alcaligenes eutrophus* and its recombinant strain. *Appl. Microbiol. Biotechnol.* **49**, 333-336.
- Halami, P. M. (2008), Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06 *World J Microbiol. Biotechnol.*, **24**, 805-812.
- Jendrossek, D.; Schirmer, A. and Schlegel, H. G. (1996), Biodegradation of polyhydroxyalkanoic acids. *Appl. Microbiol. Biotechnol.*, **46**, 451-463.
- Kessler, B. and Witholt, B. (2001), Factors involved in the regulatory network of polyhydroxyalkanoate metabolism. *J Biotechnol.*, **86**, 97-104.
- Kim, B. S. (2007), Production of PHB from inexpensive substrates. *Enzyme Microb. Technol.*, **27**, 774-777.
- Lara, L.; Madison. and Huisman, G. W. (1999), Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic, *Microbiol. Mol Biol. Rev.*, **63**, 21-53
- Lee, S.Y. (1996), Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. *Trends Biotechnol.*, **14**, 431-438.
- Lowry, O. H.; Rosenbough, H. I.; Fair, A. L. and Randall, R. I. (1951), Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.
- Mercan, N. and Beyatli, Y. (2001), Production of poly-β-hydroxybutyrate (PHB) by *Bacillus sphaericus* strains. *J. Biotechnol* **25**, 2, 1-7.
- Mergaert, J.; Anderson, C.; Wouters, A. and Swings, J. (1994), Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in compost. *J. Environ. Polym Degrad.*, **2**, 177-183.
- Mergaert, J.; Webb, A.; Anderson, C.; Wouters, A. and Swings, J. (1993), Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils, *Appl. Microbiol. Environ. Microbiol.*, **59**, 233-3238.
- Miller, G. L. (1959), Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**, 426-428.
- Nam, D. H. and Ryu, D. D. Y. (1985), Relationship between butirosin biosynthesis and sporulation in *Bacillus circulans*. *Antimicrob. Agents Chemother.* **27**, 789-801.
- Nonato, R. V.; Mantelatto, P. E. and Rossell C. E. V. (2001), Integrated production of biodegradable plastic, sugar and ethanol, *Appl. Microbiol. Biotechnol.*, **57**, 1-5.

- Oliveira, F. C.; Freire D. M. G. and L.R. Castilho, L. R. (2004), Production of poly(3-hydroxybutyrate) by solid-state fermentation with *Ralstonia eutropha* *Biotechnol. Lett.*, **26**, 1851-1855.
- Rusendi, D. and Sheppard, J. D. (1995) Hydrolysis of potato processing waste for the production of poly- $\beta$ -hydroxybutyrate. *Bioresour. Technol.*, **54**, 191-196.
- Slepecky, R. A. and Law, J. H. (1961), Synthesis and degradation of poly- $\beta$ -hydroxybutyric acid in connection with sporulation of *Bacillus megaterium*. *J. Bacteriol.* **82**, 37-42.
- Slepecky, R. A. and Law, J. H. (1960), A rapid spectrophotometric assay of  $\alpha$ ,  $\beta$ -unsaturated acids and  $\beta$ -hydroxy acids. *Anal. Chem.*, **32**, 1697-1699.
- Sudesh, K.; Abe, H. and Doi, Y. (2000), Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog Polym Sci.*, **25**, 1503-1555.
- Taidi, B.; Anderson, A. J.; Dawes, E. A. and Byrome, D. (1994), Effect of carbon source and concentration on the molecular mass of poly(3-hydroxybutyrate) produced by *methylobacterium extorquens* and *Alcaligenes eutrophus*. *Appl. Microbiol. Biotechnol.* **40**, 786-790.
- Thakor, N.; Trivedi, U. and Patel, K. C. (2005), Biosynthesis of medium chain length poly(3-hydroxyalkanoates) (mcl-PHAs) by *Comamonas testosteroni* during cultivation on vegetable oils *Bioresour. Technol.*, **96**, 1843-1850.
- Van- Thuoc, D.; Quillaguaman, J.; Mamo, G. and Mattiasson, B. (2007), Utilization of agriculture residues for poly(3-hydroxybutyrate) production by *Halomonas boliviensis* LC1. *J. Appl. Microbiol.*, **104**, 420-428.
- Yilmaz1, M.; Soran, H. and Beyatli, Y. (2005), Determination of poly- $\beta$ -hydroxybutyrate (PHB) production by some *Bacillus* spp. *World J. Microbiol. Biotechnol.*, **21**, 565-566.

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