International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.4, No.3, pp 1158-1162, July-Sept 2012

Polyhydroxybutyrate (PHB) production using agro-industrial residue as substrate by Bacillus thuringiensis IAM 12077.

Srividya Shivakumar

Department of Microbiology, Centre for PG Studies, Jain University, 18/3, 9th Main, 3rd Block Jayanagar, Bangalore 560011,India.

Corres.author : sk2410@yahoo.co.uk; sk.srividya@jainuniveristy.ac.in
Tel: +91080 43226500;

Abstract: The aim of this work was to study the production of polyhydroxybutyrate (PHB) using pure glucose and agro- industrial residues, as the carbon source**.** The optimum conditions for PHB production with pure glucose by *B.thuringiensis* IAM 12077 was found to be pH 7.5, inoculum density of 200% and 24h of incubation in the production phase. Eight substrates, viz., soya fluor, CMC, bagasse, molasses, wheat bran , wheat germ, rice bran and ragi bran, were assessed as alternative cheap substrates for PHB production. Maximum production was obtained with 10g/L soya powder fermented for 24 h at 30^{o}C in a medium containing nutrient salts and initial pH as 7.5. The PHB content in the cells was 0.89g/L and 25.28% which was comparable to the PHB yield and accumulation by this strain on pure glucose (0.81g/L; 37.17%).
Key words: PHB production, *B.thuringiensis* IAM12077, glucose, agrowastes.

INTRODUCTION

PHA are biodegradable, water insoluble, non-toxic, bio-compatible, piezoelectric, thermoplastic, and/or elastomeric. These features make them suitable for applications in the packaging industry and as substitute for hydrocarbon-based plastics[1]. 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[2]. The low molecular weight P (3HB) is a part of bacterial Ca2+ channels[3]. 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[4-6]. 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[7]. Both the shortening of external nutrients and internal sources such as RNA or enzymes facilitate the PHA synthesis.

Currently, research on PHB has centered around making its production economic so as to compete with petrochemical derived polymers. In PHA production by fermentation, the substrate and recovery costs are high, making their use unattractive. Carbon source for PHB production accounts up to 50% of the total production costs. Thus, the use of waste agricultural residues, starch and dairy waste can substantially reduce the substrate cost (and in turn even provide value to the waste), and can downsize the production costs. This improves the market competitiveness through considerable cost savings over the prior art practice of using rich medium with glucose for producing PHB[8,9]. PHA production from wastes like starch, whey, molasses, CSL, bagasse, soyameal etc. can provide cost effective as well as environmentally friendly biodegradable polymer. Very little work has been reported on the use of such wastes by PHA producing bacteria[10].

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 used in the study

PHB accumulating *Bacillus thuringiensis* IAM 12077 isolated in the previous study was used[11,12].

Production of PHB under nutrient broth and biphasic growth conditions using glucose substrate

24 h Nutrient broth grown culture of *Bacillus thuringiensis* IAM 12077 was centrifuged at 8000rpm for 10-15 min and the culture pellet was transferred to N_2 deficient medium (pH 7.0) containing 1%lactose, 0.02%$MgSO_4$, 0.01% NaCl, 0.05% KH_2PO_4, 0.25% peptone, and 0.25% yeast extract[10,11]. Production studies were carried out in 250 ml flasks containing 50 ml culture medium and incubated at 37°C on a rotatory shaker at 120 rpm for 48h. To make a solid medium, 1.5% agar was added to the broth. The PHB production in biphasic growth condition was performed with glucose (1%, w/v)[13]. Further, PHB production as a function of time was determined in the second phase of growth.

Effect of cell density on PHB production

Different inoculum size varying from 10% to 500% in the first phase of growth was tested for the effect of cell density on PHB production.

Effect of pH on PHB production

Different initial pH of the medium (6.5 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.

Extraction and determination of PHB

After 48 h incubation at 37°C, 5 ml of the culture was taken and centrifuged at 8000 rpm for 15 min. The supernatant was discarded and the pellet was treated with 5 ml of sodium hypochlorite and incubated at 30°C for 2 h. After incubation, the mixture was centrifuged at 10,000 rpm for 15 min and then washed with distilled water, acetone, methanol and diethyl ether respectively for washing and extraction. (Finally the residue was extracted with boiling chloroform and filtered through Whatman No. 1 filter paper. The chloroform extract was evaporated to dryness[2]. Determination of PHB was performed routinely by dry weight estimation. The ultraviolet (UV) absorption spectrum of the polymer was analyzed following its conversion to crotonic acid by treatment with concentrated H_2SO_4, and the absorbance was scanned between 200 and 300 nm with Elico SL150 UV-VIS spectrophotometer. For dry weight estimation, the pellet after extraction was dried to constant weight.

Cell dry weight

After centrifugation of the culture medium, the supernatant was discarded and the cell pellet was washed with distilled water. The washed pellet was re-suspended in 1 ml distilled water, transferred to pre weighed boats and dried to constant weight at 60°C. The dry weight of the cells was determined by drying the washed cells to constant dry weight.

Evaluation of agro-industrial residues as substrate

24 h Nutrient broth grown culture of *Bacillus thuringiensis* IAM 12077 was centrifuged at 8000rpm for 10-15 min and the culture pellet was transferred to N_2 deficient medium (pH 7.0) containing 1% (w/v) of the eight different carbon sources like soya fluor, CMC, bagasse, molasses, wheat bran , wheat germ, rice bran and ragi bran, respectively, 0.02%$MgSO_4$, 0.01% NaCl, 0.05% KH_2PO_4, 0.25% peptone, and 0.25% yeast extract[10]. Production studies were carried out in 250 ml flasks containing 50 ml culture medium and incubated at 37°C on a rotatory shaker at 120 rpm for 48h.

Fig.1. PHB production of *B. thuringiensis* IAM12077 as a function of incubation time.

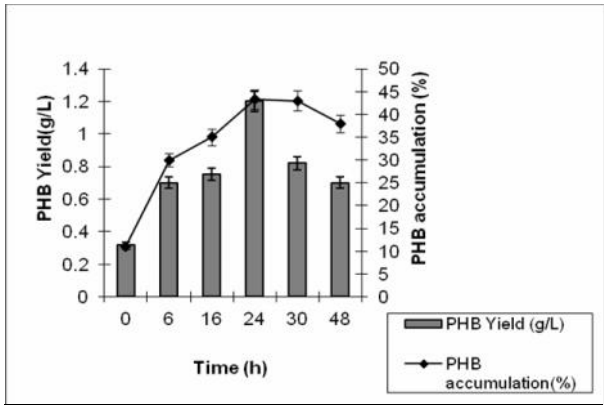


Fig.2. Effect of cell density on PHB production by *B.thuringiensis* IAM12077.

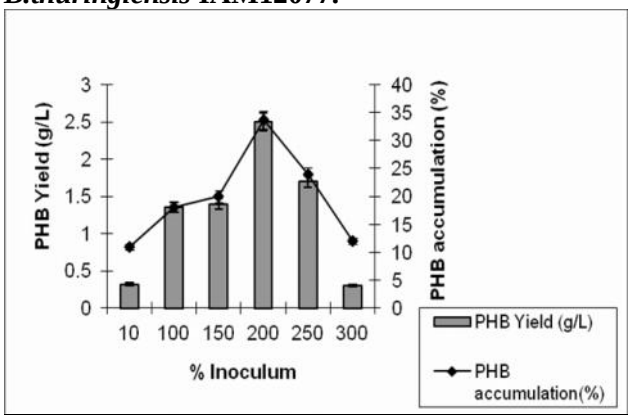


Fig.3. Effect of medium pH on PHB production by *B.thuringiensis* IAM12077.

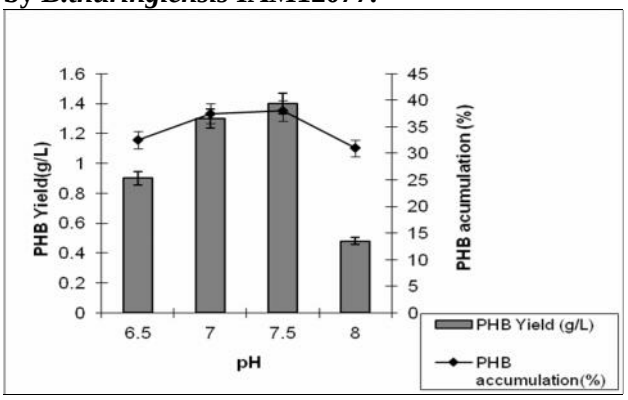
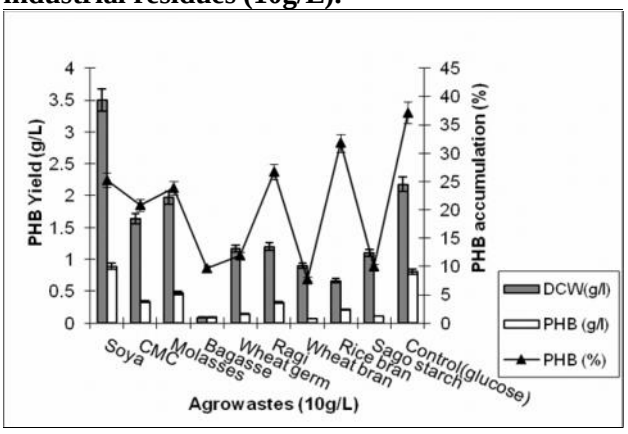


Fig.4. Comparison of synthesis of PHB by *B.thuringiensis* IAM12077 from different agro-industrial residues (10g/L).



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 glucose was 1g/L. Fig. 1 shows the PHB production at particular intervals. The PHB yield and accumulation increased until 24th of fermentation and were 43.33 % and 1.2g/L, respectively. The slight decrease in PHB production after 30h 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[14,15]. Yilmaz et al., (2005)[16] reported that some *B. sphaericus* strains were able to synthesize PHB up to 32.55% (w/v). Yuksekdag (2004) [17] reported *B.megaterium* 12 and *B.subtilis* 25, both to have produced maximum PHB at the 45th hour of incubation followed by a similar decline. Our strain achieves maximum level of PHB within 24h under similar growth conditions, making it a more industrially viable strain with respect to faster productivity.

Effect of cell density on PHB production

Cell biomass increased with increasing cell density from 10% to 300 % in the biphasic growth condition (2.85g/L to 7.4g/L) with concomitant increase in PHB production (0.32 g/L, 11% to 2.5g/L, 33.7%) after which the cell dry biomass started showing a decrease upto 2.4g/L with decrease in PHB production from 2.5g/L to 0.3 g/L at 300% inoculum (Fig.2). *Alcaligenes latus* has also been shown to produce more cell mass as well as PHB in cultures of higher cell density[18]. *Methylobacterium extorquens* ATCC 55366 has been successfully grown in high cell density cultures with accumulation reaching 46%[19]. We observe a similar trend in this study where a higher cell density yields higher PHB content upto 200% inoculums density beyond which the effective nutrient concentrations must be limiting.

Effect of medium pH on PHB production

pH of the medium from 6.5 to 8.0 supported PHB production by this strain ranging from 32.5 % to 38 %; 0.9g/L to 1.4g/L) (Fig.3). *Alcaligenes eutrophus* MTCC 1285 is reported to produce more PHB at pH

8.0 as compared to pH 6.9[13]. Grothe et al. (1999)[20] have shown that in *A.latus* the optimum pH supporting PHB production was found to be 6.5. A similar effect was found in *Rhodospirullum rubrum* where the optimal pH lay between pH 7.0 and 8.0[21].

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. To confirm the feasibility of using agro-wastes to replace glucose in the production of PHB by *B.thuringiensis* IAM 12077, dry cell weight and PHB accumulation were determined on different carbon sources like soya fluor, CMC, bagasse, molasses, wheat bran , wheat germ, rice bran and ragi bran, in shake flask cultures. Fig.4 shows the PHB production ability of the organism in the order soya (0.89g/L, 25.28%) › molasses (0.47g/L, 23.81%) › ragi bran (0.32g/L; 26.66%) › CMC (0.34g/L, 20.79%) › rice bran (0.21g/L;31.81%) ; › wheat germ (0.14g/L, 11.96%) sago starch (0.11g/L; 10%) with least growth and PHB production in bagasse (0.09g/L, 9.68%) and wheat bran (0.07g/L; 7.7%).

Van-Thuoc et al., (2007) reported 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[22]. Pandey et al., (2009)[23] have reported PHB production by *Bacillus sphaericus* NCIM 5149 using such processed agrowastes. In this study, we report PHB production of similar levels using amylolytic *B.thuringiensis* IAM 12077 from agrowastes which circumvents the hydrolysis step of agrowaste processing as the extracellular amylase of the strain itself will help utilization of such wastes directly for bioconversion into PHB.

Several agro-industrial residues such as potato starch, babassu, soy cake 24, cane molasses, whey[25] 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[26]. Thakor et al. (2005)[27] found the coconut oil as one of the best carbon source for *Comamonas testosteroni*. Rusendi and John (1995)[28] used waste potato starch hydrolyzate for the production of PHB and reported a yield of 77% of the biomass dry weight. Pandey et al. (2009)[23] reported 0.710g/L, 47% PHB accumulation with potato starch and 0.690g/L, 46% PHB accumulation with Jackfruit seed powder. *Bacillus* species also has been reported to have the capacity to produce PHAs from soy molasses. It has been reported to accumulate upto 90%, utilizing soy molasses oligosaccharides like raffinose without the need for nutrient limitation[29].

CONCLUSION

From the studies it was concluded that agricultural wastes could be an alternative option for PHB production by *B.thuringiensis* IAM12077. Maximum production was obtained with 10g/L soya powder fermented for 24 h at 30^{o}C in a medium containing nutrient salts and initial pH as 7.5. The PHB content in the cells was 0.89g/L and 25.28% which was comparable to the PHB yield and % accumulation by this strain on pure glucose (0.81g/L; 37.17%). Further optimization studies with some of these agro-wastes for PHB production will definitely throw light upon the possibility of using this strain to develop cost-effective biopolymer.

ACKNOWLEDGEMENT

The authors wish to express their gratitude for the support extended by the management of Jain University, Bangalore, India in carrying out the research work in the Department of Microbiology.

REFERENCES

1. Anderson, A.J., Dawes, E. A., Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates, 1990, Microbiol Rev., 54, 450-472.
2. Slepecky, R. A., Law, J. H., A rapid spectro photometric assay of α, β-unsaturated acids and ,β-hydroxyacids, 1960, Anal.Chem., 32, 1697-1699.
3. Lara, L., Madison, Huisman, G.W., Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic, 1999, Microbiol. Mol Biol. Rev., 63, 21-53
4. Lee, S.Y., Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria,1996,Trends Biotechnol.,14 431-438.
5. Sudesh, K., Abe, H., Doi, Y., 2000, Synthesis, structure and properties of polyhydroxy alkanoates: biological polyesters. 2000, Prog Polym Sci., 25, 1503-1555.

6. Kessler, B., Witholt, B., Factors involved in the regulatory network of polyhydroxyalkanoate metabolism, 2001, J Biotechnol., 86, 97-104.
7. Dawes, E.A.,Senior,P.J., The role and regulation of energy reserve polymers in microorganisms, 1973, Adv. Microbiol. Physiol. 10, 135-266.
8. Halami, P. M., Production of polyhydroxy alkanoate from starch by the native isolate *Bacillus cereus* CFR06, 2008, World J Microbiol. Biotechnol., 24, 805-812.
9. Lee, S.Y. *Biotechnol. Bioeng*., Bacterial Polyhydroxyalkanoates, 1996, 49, 1-14.
10. Mercan, N., Aslım, B., Yüksekdağ, Z.N., Beyatli, Y., Production of poly- β-hydroxy butyrate (PHB) by some Rhizobium bacteria, 2002, Turk J. Biol., 26, 215-219.
11. Adwitiya, P., Ashwini, P., Avinash, A.K., Badri, R., Kajal, K., Vomsi P., Srividya S., 2009, Pol.J Microbiol., 58(2), 149-154.
12. Srividya, S., Production of Poly-3-hydroxybutyrate (PHB) from lactose and whey by *Bacillus thuringiensis* IAM 12077, 2011, Res J Biotech., 6(4), 12-18.
13. Senthil, K.B., Prabakaran, G., Production of PHB (bioplastics) using bio-effluent as substrate by *Alcaligens eutrophus*, 2006, Ind J Biotechnol., 5,76-79.
14. Benoit, T. G., Wilson, G. R., Baygh, C. L., Fermentation during growth and sporulation of *Bacillus thuringiensis* HD-1, 1990, Lett. Appl. Microbiol., 10, 15-18.
15. Nam, D. H., Ryu, D. D. Y., Relationship between butirosin biosynthesis and sporulation in *Bacillus circulans*, 1985, Antimicrob. Agents Chemother., 27, 789-801.
16. Yilmaz, M., Soran, H., Beyatli, Y., Determination of poly-*b*-hydroxybutyrate (PHB) production by some *Bacillus* spp., 2005, World J. Microbiol. Biotechnol., 21, 565-566.
17. Yuksekdag, Z.N., Belma, A., Yavuz, B., Nazime, M., Effect of carbon and nitrogen sources and incubation times on PHB synthesis by *Bacillus subtilis* 25 and *B.megaterium* 12, 2004, African J Biotechnol., 3(1), 63-66.
18. Yamane, T., Fukunga, M., Lee, Y.W., Increased PHB productivity by high-cell-density fed-batch culture of *Alcaligenes latus*, a growth-associated PHB producer, 1996, Biotechnol. Bioeng., 50, 197-202.
19. Bourque, D., Pomerleau, Y., Groleau, D., High-cell- density production of PHB from methanol by *Methylobacterium extorquens*: production of high molecular mass PHB, 1995, Appl. Microbiol. Biotechnol., 44: 367-376.
20. Grothe, E., Moo-Young, M., Chisti, Y., Fermentation optimization for the production of PHB microbial thermoplastic, 1999, Enz. Microbiol. Technol., 25, 132-141.
21. Hashimoto, K., Tsuboi, H., Iwasaki, S., Shirai, Y., Effect of pH on the production of poly (β) hydroxybutyrate by photosynthetic bacterium, *Rhodospirillum rubrum*, 1993, J Chem. Eng. of Japan, 1993, 26, 56-58.
22. Van- Thuoc, D., Quillaguaman, J., Mamo, G., Mattiasson, B., Utilization of agriculture residues for poly (3-hydroxybutyrate) production by *Halomonas boliviensis* LC1, 2007, J. Appl. Microbiol., 104, 420-428.
23. Pandey, A., Nisha, V., Ramadas, Sudheer K. S., Carlos R. S., Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149, 2009, Braz. Arch. Biol. Technol., 52(1), 17-23.
24. Oliveira, F. C., Freire D. M. G., Castilho L.R., Production of poly (3-hydroxybutyrate) by solid-state fermentation with *Ralstonia eutropha*,2004,Biotechnol. Lett., 26, 1851-1855.
25. Ahn, W. S., Park, S. J., Lee, S. Y., Production of poly(3-hydroxybutyrate)from whey by cell recycle fed-batch culture of recombinant *Escherichia coli*, 2001, Biotechnol. Lett., 23, 235-240.
26. Fukui, T., Doi, Y., Efficient production of polyhydroxyalkanoates from plant oils by *Alcaligenes eutrophus* and its recombinant strain, 1998, Appl.Microbiol. Biotechnol., 49, 333-336.
27. Thakor, N., Trivedi, U., Patel, K. C., Biosynthesis of medium chain length poly (3-hydroxyalkanoates) (mcl-PHAs) by *Comamonas testosteroni* during cultivation on vegetable oils, 2005, Bioresour. Technol., 96, 1843-1850.
28. Rusendi, D., Sheppard, J. D., Hydrolysis of potato processing waste for the production of poly-β-hydroxybutyrate, 1995, Bioresour. Technol., 54, 191-196.
29. Full, T.D., Jung, D.O., Madigan, M.T., Production of poly-*β*-hydroxyalkanoates from soy molasses oligosaccharides by new, rapidly growing *Bacillus* species, 2006, Lett. Applied Microbiol., 43, 377-384.

*****