Polish Journal of Microbiology
2009, Vol. 58, No 2, 149–154

ORIGINAL PAPER

Optimization of Process Parameters for Maximum Poly(-β-)hydroxybutyrate (PHB) Production by *Bacillus thuringiensis* IAM 12077

ADWITIYA PAL, ASHWINI PRABHU, AVINASH ARUN KUMAR, BADRI RAJAGOPAL, KAJAL DADHE, VOMSI PONNAMMA and SRIVIDYA SHIVAKUMAR*

Department of Microbiology, Center for PG Studies, SBM Jain College, Jayanagar, Bangalore

Received 19 January 2009, revised 20 March 2009, accepted 2 April 2009

A b s t r a c t

The study aimed at screening and identifying a potential poly-β-hydroxybutyrate (PHB) accumulating *Bacillus* strain and optimization of media parameters for increased PHB production by the strain. A Gram-positive bacterium that accumulated PHB was isolated from local garden soil of Bangalore. Based on morphological and physiological properties, and nucleotide sequence (about 1.5 kb) of its 16S rDNA it was identified as *Bacillus thuringiensis* IAM 12077. PHB production was found to be comparable to most of the *Bacillus* sp. reported to date. PHB production by this strain was dependent on nutrient limitation. Cell dry weight and PHB accumulation increased significantly under biphasic growth condition (from nutrient broth to nitrogen-deficient medium) as compared with growth in nutrient broth alone (from 0.32 g/l to 2.76 g/l cell dry weight; 24% to 43.37% PHB accumulation; 0.2 g/l to 1.2 g/l PHB production), with maximum accumulation at 24 h in nitrogen-deficient medium. Time course study of growth and PHB production by this strain in the nitrogen deficient medium showed that PHB production was associated with the stationary phase of growth. All the tested media containing different carbon and nitrogen sources supported growth and PHB production. Ultraviolet spectrum of the extracted polymer showed a characteristic peak at 235 nm.

K e y w o r d s: *Bacillus* sp., biphasic studies, carbon and nitrogen sources, fermentation kinetics, Poly(-β-)hydroxybutyrate

Introduction

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyacids naturally synthesized by bacteria as a carbon reserve. PHAs have properties of biodegradable thermoplastics and elastomers and their synthesis is seen as an attractive system for the sustained production of large amounts of polymers at low cost (Ojumu *et al.*, 2004; Madison and Huisman, 1999; Fiechter, 1990). PHAs accumulate as cytoplasmic inclusions in certain bacteria during unbalanced growth conditions, usually characterized by an excess supply and the lack of one or more essential nutrients. Poly(-β-)hydroxybutyrate (PHB) is the best known PHA. PHB is the alternative source of the plastics which has similar physical properties like polypropylene and it can be easily biodegradable aerobically and an-aerobically.

PHB and its copolymers have been industrially produced since 1982 as substitutes for petroleum based plastics. Global environment concerns and solid waste management problems have generated much interest in the development of biodegradable plastics that retain the desired physical and chemical properties of the conventional synthetic plastics. PHAs have found wide ranging applications as biodegradable and biocompatible polymers. However, the general substitution of conventional plastics has been limited by high production costs (Ojumu *et al.*, 2004). Therefore, more efforts should be devoted to make this process economically feasible by further understanding PHB accumulation process and improving the productivity. In biotechnological aspects, cheap substrates, mutations and genetically modified high PHB yielding bacteria (or) plants can be used in biopolymer production technique.

A number of *Bacillus* species have been reported to accumulate 9–67% dry cell weight PHA (Anderson *et al.*, 1990; Hikmet *et al.*, 2003; Hori *et al.*, 2002; Lach *et al.*, 1990; Mercan *et al.*, 2002; Page, 1989; Rohini *et al.*, 2006; Wu *et al.*, 2001; Ramsay *et al.*, 1990; Wakisaka, 1982). *Bacillus thuringiensis*, better known for its insecticidal δ-endotoxin, has been reported to accumulate PHB (Belma *et al.*, 2002; Senthil

* Corresponding author: S. Srividya, Department of Microbiology, Center for PG Studies, SBM Jain College, 18/3, 9th Main, 3rd block Jayanagar, Bangalore-560011; phone: (+91) 080 41210691; fax: (+91) 080 41210692; **e-mail: sk2410@yahoo.co.uk**

and Prabakaran, 2006). In this paper we report the PHB accumulation ability by *Bacillus thuringiensis* IAM 12077 and also describe the influence of nutritional conditions on its growth and PHB accumulation.

Experimental

Materials and Methods

Isolation of PHB producing *Bacillus* sp. Different soil samples were collected from in and around Bangalore in the month of July. Screening to collect spore-forming bacteria was performed by heating samples at 80°C for 10 minutes and then plating them on nutrient agar (NA). The resulting bacterial colonies were tested for PHA accumulation by staining with sudan black (0.3% in 96% ethanol) (Belma *et al.*, 2002). Bluish-black colonies indicating PHB production were characterized. Different PHB positive *Bacillus* sp. identified as Gram-positive spore forming rods were chosen for further studies.

Characterization of the isolated bacteria. The morphological and physiological properties of the isolate were investigated according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1993). The sequencing of 16S rDNA and taxonomic studies of strain IAM 12077 were performed at The Chromous Biotech. Pvt Ltd. Bangalore India. A partial 16S r DNA fragment (~1.5 kb) was amplified using high-fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the forward reverse and internal primers. The sequence data was aligned and analyzed to identify the bacterium and for finding nearest related strains.

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 7155.2 xg for 10–15 min and the bacterial pellet was transferred to N_2 deficient medium (pH 7.0) containing 1% glucose, 0.02% $MgSO_4$, 0.01% NaCl, 0.05% KH_2PO_4, 0.25% peptone, and 0.25% yeast extract (Mercan *et al.*, 2002). 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 48 h. To make a solid medium, 1.5% agar was added to the broth. The PHB production in biphasic growth condition was compared with nutrient broth growth at various time intervals (Senthil and Prabakaran, 2006). Next, PHB production as a function of time was determined in the second phase of growth.

Extraction and determination of PHB yield. After 48 h incubation at 37°C, 5 ml of the culture was collected and centrifuged at 8000 xg 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 × g 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 (Law and Slepecky, 1961). Determination of PHB yield 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 resuspended in 1 ml distilled water, transferred to pre weighed boats and dried to constant weight at 60°C.

Effect of different carbon and nitrogen sources on PHB production. Glucose in the N_2 deficient medium was replaced by 1% starch, arabinose, mannose, galactose, xylose, sucrose and lactose respectively as carbon sources and yeast extract was replaced by 0.25% peptone, tryptone, casein, $(NH_4)_2SO_4$, NH_4Cl and $NaNO_3$ as nitrogen source.

Statistical analyses. The mean and standard deviation were calculated from at least two independent experiments in duplicate.

Results

Of the total 66 *Bacillus* sp. screened from different soil samples, we selected the isolate which accumulated 0.433 g/l PHB with 20.63% yield, under biphasic growth conditions, after 48 h. The morphological and taxonomical features of the isolate were examined (Table I). Based on the biochemical characteristics, the isolate was characterized and identified as the endospore forming, Gram-positive *Bacillus thuringiensis*. Analysis of the partial nucleotide sequence of the 16S rDNA (~1.5 kb) identified the isolate to be *B. thuringiensis* IAM 12077 with a close homology to *Bacillus cereus* IAM 12605 (data not shown).

Poly-β-hydroxybutyrate (PHB) production by *B. thuringiensis* IAM 12077 was investigated at biphasic growth conditions. Cell dry weight and PHB accumulation increased significantly under biphasic growth condition (from nutrient broth to nitrogen-deficient medium) as compared with growth in nutrient broth alone (from 0.32 g/l to 2.76 g/l cell dry weight; 24% to 43.33% PHB accumulation; 0.2 g/t to 1.2 g/l

Table I
Physiobiochemical characteristics of the isolate *Bacillus thuringiensis* IAM 12077

Test	Observation
Gram's stain	Gram positive
Spore staining	Central, oval, bulging
Cell shape	Rods
Cell size	>3 µm
Colony character	White, raised, irregular
Motility	+
Catalase	+
Oxidase	–
Indole	–
Methyl Red	–
Voges-Proskauer	–
Citrate utilization	+
Casein Hyrdolysis	+
Aesculin hydrolysis	+
Starch hydrolysis	+
Urea hydrolysis	–
Growth at 50°C	+
Growth in 10% NaCl	–
Anaerobic growth	+
TSI	Acid slant/Alkaline butt, gas, no H_2S
Sugar Utilization	**Result (Acid/Gas)**
Glucose	+/–
Galactose	+/–
Arabinose	+/–
Mannitol	–/–
Maltose	+/–
Mannose	+/–
Raffinose	–/–
Rhamnose	–/–
Sucrose	+/–
Salicin	+/–
Lactose	+/–
Fructose	+/–
Xylose	+/–

+ positive; – negative

PHB production) with maximum accumulation at 24 h in biphasic condition (Table II).

The time-course of aerobic growth and PHB accumulation was measured at different time intervals after transfer into the nitrogen deficient medium (Fig. 1).

Table II
Biphasic growth studies in *Bacillus thuringiensis* IAM 12077

Media	PHB accumulation (%)		PHB Yield (g/l)	
	24 h	48 h	4 h	48 h
1. Nutrient Broth (NB)	10.52	24	0.066	0.2
2. Biphasic (NB to N_2 deficient medium)	43.33	38.28	1.2	0.58

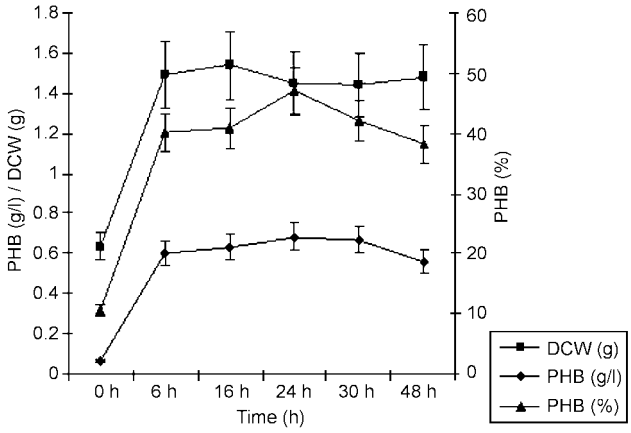

Fig. 1. Growth and poly(-β-)hydroxybutyrate (PHB) accumulation by the strain of *Bacillus thuringiensis* IAM 12077 in production medium containing 10g glucose per liter.

Cell mass increased by 16 h from 0.633 g/l to 1.4 g/l and maintained steadily, PHB production also gradually increased, attained its maximum at 24 h of cultivation (from 0.066 g/l to 0.683 g/l), with a gradual increase in PHB yield and accumulation with maximum at 24 h (0.683 g/l, 47.1%), after which the PHB yield gradually decreased by 48 h.

The strain exhibited nutritional versatility in terms of varied growth and PHB production when tested on the various carbon and nitrogen sources. The highest levels of PHB accumulation (g/l) was observed in the N_2-deficient medium with xylose (cell dry biomass 2.9 g/l; PHB accumulation 41.3%; 1.2 g/l PHB yield) followed by lactose (1.06 g/l), sucrose (0.9 g/l), mannose, starch (0.7 g/l), arabinose (0.66 g/l), glucose (0.46 g/l) and galactose (0.3 g/l). Of the various organic and inorganic nitrogen sources tested, all sources supported cell growth and PHB production in the order peptone >casein >yeast extract (YE) >tryptone among

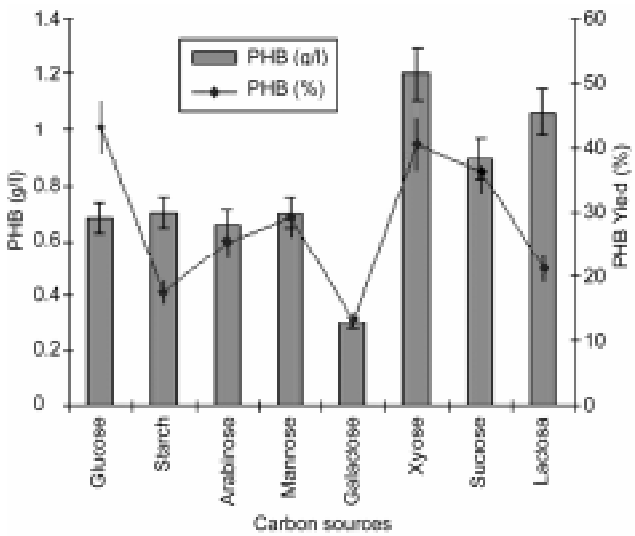

Fig. 2a. Effect of different carbon sources on PHB yield and accumulation in *Bacillus thuringiensis* IAM 12077.

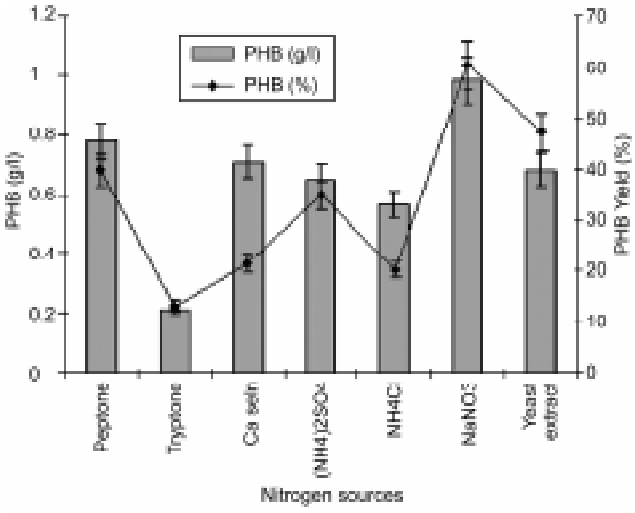

Fig. 2b. Effect of different nitrogen sources on PHB yield and accumulation in *Bacillus thuringiensis* IAM 12077.

Table III
Fermentation kinetic parameters of the strain *Bacillus thuringiensis* IAM12077 with xylose and $NaNO_3$ supplementations in place of glucose and YE, respectively in the nitrogen deficient media

Parameters	Values	
	Xylose:YE	Glucose:$NaNO_3$
PHB (%)	40.48	60.3
PHB yield ($Y_{p/s}$)	0.12	0.098
Q_p	0.05	0.04
q_p	0.016	0.025

$Y_{p/s}$ PHB yield at the end of fermentation (g_{PHB}/g_s)
Q_p Volumetric rate of poly (β)hydroxybutyrate (PHB) formation (g_{PHB}/l/h).
q_p specific rate of PHB formation (g_{PHB}/g_x/h)
Q_p Volumetric rate of poly (β)hydroxybutyrate (PHB) formation (g_{PHB}/l/h).
$Y_{p/s}$ PHB yield at the end of fermentation (g_{PHB}/g_s)

the organic nitrogen sources and $NaNO_3$ > $(NH_4)_2SO_4$ >NH_4Cl among the inorganic nitrogen sources. $NaNO_3$ supported highest PHB yield and accumulation (cell dry biomass 1.63 g/l; PHB accumulation 60.3%, 0.983 g/L PHB yield) (Fig. 2a and 2b). The highest levels of PHB production was observed in the nitrogen-deficient medium with xylose (1.2 g/l) as the carbon source, while $NaNO_3$ supported maximum accumulation (60.3%) when used as nitrogen source. Of the various carbon and nitrogen supplements tested for PHB production, both xylose: YE and glucose: $NaNO_3$ medium combinations increased PHB production and productivity PHB (g/l); Qp (g_p/l/h) and $Y_{p/s}$] (Fig. 4). Addition of xylose in the N_2 deficient medium increased the values of PHB yield (g/l) by 1.46 fold, while replacement of YE in the medium by $NaNO_3$ increased both PHB (% DCW) and PHB yield (g/l) by 1.43 fold and 1.28 fold, respectively. Fermentation parameters are summarized in Table III and Fig. 3.

The purified polymer was highly soluble in chloroform, 1 N NaOH; moderately soluble in dioxane, pyridine, and toluene, but insoluble in water, sodium hypochlorite, acetone, ethanol, methanol, and diethyl ether. Digestion of the polymer with concentrated H_2SO_4 gave a sharp peak at 235 nm characteristic of crotonic acid (Fig. 4).

Discussion

B. thuringiensis, better known for its δ-endotoxin has been reported to accumulate PHB. However, only one study has been reported to gauge the influence of media (carbon) and culture conditions on the accu-

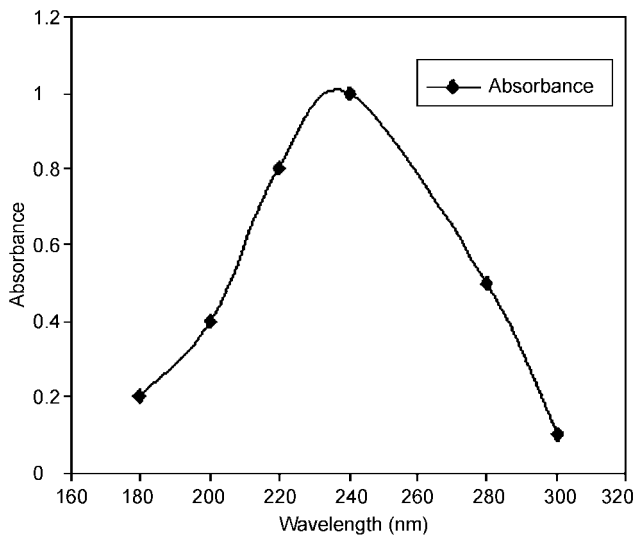

Fig. 5. Ultraviolet absorption spectra of isolated PHB following depolymerization and dehydration in concentrated sulfuric acid.

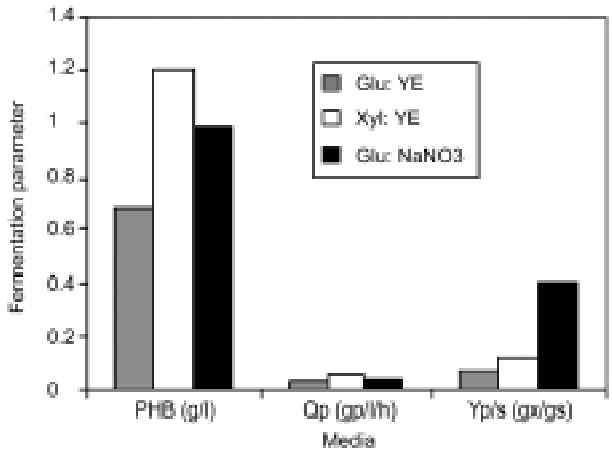

Fig. 4. Fermentation kinetic parameters of the strain *Bacillus thuringiensis* IAM 12077 grown in batch culture under optimal conditions of cultivation.

mulation of PHAs by *B. thuringiensis*. The present study highlights the accumulation of PHB by *B. thuringiensis* IAM 12077 and optimization of media for maximizing PHB production by this strain.

Bacteria able to synthesize PHA can be divided into two groups. The first group, accumulating PHA during the stationary phase, requires limitation of N, P, Mg and oxygen, for example, and an excess of the carbon sources. The most important microorganism for industrial PHA production, *Ralstonia eutropha*, belongs to this group. The second group, accumulating PHA during the growth phase, includes *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii*, *A. beijerinckii* (Borah *et al.*, 2002) or recombinant strains of *E. coli* bearing the PHA operon of *R. eutropha*. Our strain, *B, thuringiensis* IAM 12077, belongs to the first group because PHB accumulates during stationary phase. Some of the *Bacillus* species have been reported to accumulate 6–36% PHB of the cell dry mass (Belma *et al.*, 2002). *B. thuringiensis* is also reported to produce 64.1% PHB in biphasic condition indicating that while the first phase is used for the development of the biomass, the second phase is preferentially utilized for PHB production by the cells (Senthil and Prabakaran, 2006). The physiological characteristics of the organism are important for the bioprocess to produce polymer.

B. thuringiensis IAM 12077 showed capability of producing PHB using simple sugars like pentoses and hexoses and polysaccharides like starch. The nutritional versatility of this strain can be explored for economic and eco-friendly PHB production using hemicelluloses, starch and whey. Borah *et al.* (2002) reported the use of sucrose as the cheaper source for the production of PHB by *Bacillus mycoides* RLJ B-07 (Borah *et al.*, 2002). In *Bacillus megaterium* PHB content in the cell reached a maximum level after growing with glucose as sole carbon source (39.9%) (Borah *et al.*, 2002). Similarly, *Bacillus* sp. Jma5 (25–35%) and *B. megaterium* (40.8%) accumulated PHB during fermentation with molasses (Gouda *et al.*, 2001; Hängii, 1995; Yuksekdag *et al.*, 2004). Belma *et al.* (2002) have reported PHB accumulation of 8.0 and 7.5% in *B. thuringiensis* strains D1 and D2, respectively in glucose containing medium. The highest level of PHB accumulation was observed in the medium with glucose as carbon source in *B. subtilis* (19.51%), *B. megaterium* (19.49%).

The highest level of PHB accumulation was observed in the medium with protease peptone as nitrogen sources in *B. subtilis* 25 (78.69%) and in *B. megaterium* 12 (77%) (Yuksekdag *et al.*, 2004). Page (1992) tested PHB production in a variety of commercially available complex nitrogen sources. It was found that complex nitrogen sources increased the yield of PHB produced by *A. vinelandii* UWD strain. Mercan *et al.* (2002) investigated the effect of different nitrogen and carbon sources and PHB production in two strains of *Rhizobium* sp. We also report varied levels of PHB production by *B. thuringiensis* IAM 12077 with different carbon and nitrogen sources in accordance with the literature. In this study, *B. thuringiensis* IAM 12077 showed highest level of PHB production (60.3%) in medium containing glucose: $NaNO_3$ combination in the ratio of 4:1 which is comparable to that reported by Rohini *et al.* (2006) for another strain of *B. thuringiensis* (64.1 %) in biphasic condition with glycerol.

Digestion of the polymer with concentrated H_2SO_4 gave a sharp peak at 235 nm characteristic of crotonic acid indicating the presence of PHB.

Gram-positive bacteria have not been reported to accumulate large amounts of polyhydroxyalkanoate and hence have not been considered as potent candidates for industrial production. A number of *Bacillus* spp. have been reported to accumulate 9–67% dry cell weight (DCW) PHB. By comparison, *B. thuringiensis* IAM 12077 produced 60.3% dry cell weight PHB in glucose: $NaNO_3$ (4:1) medium. The PHB yields could also be increased by media optimization and high cell densities. The capability of the strain to utilize pure sugars of hemicelluloses, starch and lactose makes it promising to produce biopolymers from agro wastes and dairy wastes.

The identity and purity of the PHB obtained from *B. thuringiensis* IAM 12077 have been confirmed by solubility properties and UV- absorption spectra.

Further optimization of the media and growth conditions with the best carbon and nitrogen concentration as well as mutagenesis for further increase in PHB production by this strain will be performed.

Acknowledgement

Financial support from the management of Jain Group of Institutions, Bangalore to carry out this work is greatly acknowledged by all the authors.

Literature

Anderson A.J., G.W. Haywood and E.A. Dawes 1990. Biosynthesis and composition of bacterial polyhydroxyalkanoates. *Int. J. Biol. Macromol.* 12: 102–105.

Belma A.Y., Zehra Nur and B. Yavuz. 2002. Determination of PHB growth quantities of certain *Bacillus sp.* isolated from soil. *Turk E. J. Biotechnol.* Special issue: 24–30.

Borah B., P.S. Thakur & J.N. Nigam. 2002. The influence of nutritional and environmental conditions on the accumulation of poly-hydroxybutyrate in *Bacillus mycoides* RLJ B-017. *J. Appl. Microbiol*. 92: 776–783.

Fiechter A. 1990. Plastics from bacteria and for bacteria: *Poly (beta-hydroxyalkanoates) as Natural, Biocompatible and Biodegradable Polyesters.* Springer-Verlag, New York, p. 77–93.

Gouda M.K., A.E. Swellam and S.H. Omar. 2001. Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. *Microbiol. Research* 156: 201–207.

Hänggi U.J. 1995. Requirements on bacterial polyesters as future substitute for conventional plastics for consumer goods. *FEMS Microbiol. Rev*. 16: 213–220.

Hikmet K., A. Belma , N.K. Zehra, M. Nazime and B. Yavuz. 2003. Production of PHB and differentiation of putative *Bacillus* mutant strains by SDS-PAGE of total cell protein. *Afri. J. Biotechnol*. 2: 147–149.

Holt J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley and S.T. Williams (ed.).1993. *Bergey's Manual of Determinative Bacteriology*, 9th edition: 559–564, Williams & Wilkins, Maryland.

Hori k., M. Kaneko, Y. Tanji, X. Xing and H. Unnu H. 2002. Construction of self-disruptive *Bacillus megaterium* in response to substrate exhaustion for PHB production. *Appl. Microbiol. Biotechnol*. 59: 211–216.

Lach D.A., V.K. Sharma and P.S. Vary. 1990. Isolation and characterization of unique division of mutant of *Bacillus megaterium*. *J. Gen. Microbiol.* 136: 545–553.

Law J.H. and R.A. Slepecky. 1961. Assay of poly β-hydroxybutyric acid. *J. Bacteriol*. 82: 32–36.

Madison L.L., and G.W. Huisman. 1999. Metabolic engineering of poly(3-hydroxyalcanoates): from DNA to plastic. *Microbiol. Mol. Biol. Rev*. 63, 21–53.

Mercan N., Aslim, Z.N. Yuksekdag and Y. Beyatli. 2002. Production of PHB by some *Rhizobium* bacteria (in Turkish). *Turk. J. Biol.* 26: 215–219.

Ojumu T.V., J. Yu and B.O. Solomon. 2004. Production of polyhydroxyalkanoates, a bacterial biodegradable polymer. *Afri. J. Biotechnol.* 3, 18–24.

Page W.J. 1989. Production of PHB by *Azotobacter vinelandii* strain UWD during growth on molasses and other complex carbon sources. *Appl. Microbiol. Biotechnol.* 31: 329–333.

Page W.J. 1992. Production of poly-β-hydroxybutyrate by *Azotobacter vinelandii* UWD in media containing sugars and complex nitrogen sources *Appl. Microbiol. Biotechnol.* 38: 117–121.

Ramsay B.A., K. Lomaliza, C. Chavarie, B. Dubé, P. Bataille and P.A. Ramsay. 1990. Production of poly-(beta-hydroxybutyric-co-β-hydroxyvaleric) acids. *Appl. Environ. Microbiol.* 56: 2093–2098.

Rohini D., S. Phadnis and S.K. Rawal. 2006. Synthesis and characterization of PHB from *Bacillus thuringiensis* R1. *Indian J. Biotechnol*. 5: 276–283.

Senthil K.B. and G. Prabakaran. 2006. Production of PHB (bioplastics) using bio-effluent as substrate by *Alcaligens eutrophus*. *Indian J. Biotechnol*. 5: 76–79.

Wakisaka Y., E. Masaki and Y. Nishimoto. 1982. Formation of crystalline δ-endotoxin or poly-β-Hydroxybutyric acid granules by asporogenous mutants of *Bacillus thuringiensis*. *Appl. Environ. Microbiol*. 43: 1473–1480.

Wu Q., H. Huang, G. Hu, J. Chen, K.P. Ho and G.Q. Chen. 2001. Production of PHB by *Bacillus* sp. Jma5 cultivated in molasses media. *Antonie Van Leeuwenhoek* 80: 111–118.

Yuksekdag N.Z., A. Belma, B. Yavuz and Nazim M. 2004. Effect of carbon and nitrogen sources and incubation times on PHB synthesis by *Bacillus subtilis* and *Bacillus megaterium*. *Afri. J. Biotechnol*. 3: 63–66.