Indian J Microbiol (March 2010) 50(1):63–69
DOI: 10.1007/s12088-010-0013-1

ORIGINAL ARTICLE

Extractability of polyhydroxyalkanoate synthesized by *Bacillus flexus* cultivated in organic and inorganic nutrient media

M. S. Divyashree · T. R. Shamala

Received: 13 May 2008 / Accepted: 17 January 2009

Abstract *Bacillus flexus* was isolated from local soil sample and identified by molecular methods. In inorganic nutrient medium (IM) containing sucrose as carbon source, yield of biomass and polyhydroxyalkanoate (PHA) were 2 g/l and 1 g/l (50% of biomass), respectively. Substitution of inorganic nitrogen by peptone, yeast extract or beef extract resulted in biomass yields of 4.1, 3.9 and 1.6 g/l, respectively. Corresponding yields of PHA in biomass was 30%, 40% and 44%. Cells subjected to change in nutrient condition from organic to inorganic, lacked diaminopimelic acid in the cell wall and the concentration of amino acids also decreased. Under these conditions the extractability of the polymer from the cells by hot chloroform or mild alkali hydrolysis was 86–100% compared to those grown in yeast extract or peptone (32–56%). The results demonstrated that growth, PHA production and the composition of cell wall of *B. flexus* are influenced by the organic or inorganic nutrients present in the growth medium. Cells grown in inorganic medium lysed easily and this can be further exploited for easier recovery of the intracellular PHA.

Keywords Polyhydroxyalkanoate · *Bacillus flexus* · Cell wall composition · Organic nutrient medium · Inorganic salts medium

M. S. Divyashree · T. R. Shamala (✉)
Department of Food Microbiology,
Central Food Technological Research Institute,
Mysore – 570020, India

E-mail: shamala_trs@yahoo.co.uk

Introduction

Polyhydroxyalkanoate (PHA), which is synthesized by various bacteria as energy reserve of the cells, has attracted worldwide attention as biodegradable thermoplastic [1]. Polyhydroxybutyrate (PHB) is a common homopolymer produced by bacteria but the presence of copolymers improves the properties of the biopolymer [2, 3]. Sodium hypochlorite is generally used to hydrolyze the cell wall to release the polymer and the polymer is further purified and recovered using solvents [4]. This leads to generation of chlorine containing effluent and also results in the reduction in the molecular size of the polymer. As an alternative, enzymatic methods have been evolved for extraction [5, 6]. Various *Bacillus* species are known to produce PHA [7–9]. But the extensive cross-linking and the thick peptidoglycan layer present in the gram-positive *Bacillus* species enables the bacterium to retain cell wall even in the presence of various hydrolytic enzymes and it is more difficult to disrupt such cells to isolate the intracellular biomolecules. The present work examines the effect of organic and inorganic nutrients on growth and PHA production by *Bacillus flexus*. Effect of nutrients on composition of cell wall and extractability of PHA by simplified methods has also been studied.

Materials and methods

Microorganism

The bacterium used in this study was isolated from local soil sample. The culture was maintained at 4°C on nutrient agar slants and subcultured once in a month.

Identification of the isolate by 16S rRNA method

The 16S rRNA gene sequence analysis, which provides a measure of genomic similarity above the level of species allowing comparisons of relatedness across the genus, has been widely used for the identification of the species. In the present study the 1.2 kb of entire 16S rRNA gene from the selected isolate was sequenced which involved, isolation and purification of chromosomal and plasmid DNA, polymerase chain reaction (PCR), preparation of competent cells, transformation of *Escherichia coli* and DNA cloning [10]. The 16S rRNA gene was amplified by PCR using the DNA of the organism to be identified as a template. Two synthetic oligonucleotide primers used were:

- GCTCTAGAGCGATTACTAGCGATTCCGACTTCG
- CGACGTCGGCTCAGGATGAACGCTGGCGGC

The sequence obtained was compared by using the basic local alignment search tool (BLAST) program from the National Center for Biotechnological Information (NCBI) gene bank for the identification of the species.

Inoculum

Inoculum was prepared by using sterile nutrient broth (10 ml) taken in test tubes. The broth was inoculated with 2–3 loops full of fresh culture from nutrient agar slants. The inoculated tubes were incubated at 30°C and 250 rpm for 24 h. The inoculum concentration used for inoculation was 2×10^4 cfu/ml.

PHA production in shake flasks

PHA production was carried out in inorganic nutrient medium (IM) in 500 ml capacity Erlenmeyer flasks in triplicate and the medium contained (g/l): $Na_2HPO_4 \cdot 2H_2O$, 2.2; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.2; sucrose, 20; pH 7.0. Standard (MTCC 2909) and isolated cultures of *B. flexus* were used in the experiment. Incubation was carried out at 250 rpm and 30°C for 72 h.

Effect of organic and inorganic nutrients on growth, PHA production and cell wall composition was studied using following media: (a) Control IM mentioned above; (b) in IM $(NH_4)_2SO_4$ was replaced by yeast extract (2.5 g/l) or peptone (5 g/l) or beef extract (2.5 g/l); and (c) complex medium (g/l): peptone, 5; yeast extract, 2.5; beef extract, 2.5; $(NH_4)_2SO_4$, 1.5; sucrose, 20; pH 7.0. Inoculated flasks were incubated at 250 rpm and 30°C for 72 h. Experiments were carried out in triplicate.

PHA production in fermentor

Fermentation was carried out in a jar fermentor (3 l capacity, Bioflo 110 New Brunswick Scientific Co., Edison NJ, USA) containing 1.8 liter of mineral medium (30 g/l of sucrose) mentioned under shake flask experiment. Inoculum (0.2 l) was prepared in IM for fermentation. Fermentation was carried out at 30°C, for 48 h, culture pH was controlled at 7 by the addition of 1 mole NaOH and 50% of air saturation. Residual sugar was analyzed by dinitrosalicylic acid method [11] in the cell free supernatant after inversion of residual sucrose with HCl.

Extraction and estimation of PHA

Biomass obtained after centrifugation of fermented broth was dried to a constant weight [9]. Various methods were used for hydrolysis of cell wall and PHA extraction: (a) sodium hypochlorite solution was used for cell hydrolysis and PHA was solubilised in chloroform [12, 13]; (b) dried biomass was refluxed with chloroform at 40°C, for 2 h. The chloroform layer was separated by filtration and extracted polymer was recovered by using hexane in 1:2 proportions and the precipitated polymer was dried at 45°C to a constant weight; and (c) dried biomass (100 mg) was suspended in water (5 ml) and pH was set to 12 by ammonia solution (25% v/v). The suspension was incubated at 50°C for 10 min. The hydrolysate was centrifuged (6,000 rpm 10 min), washed with acetone, dissolved in chloroform and insoluble material was removed by filtration. Chloroform layer containing PHA was evaporated to dryness and weighed.

Identification of polymers

Gas liquid chromatography (GC) analysis of PHA was also carried out after formation of methylesters [14]. Standard polyhydroxybutyrate-co-hydroxyvalerate-P(HB-co-HV) obtained from Sigma-Aldrich, USA, was used for calibration and benzoic acid was used as an internal standard.

Cell wall analysis

Cell wall was isolated according to reported method [15]. The purified pelleted cell wall obtained was lyophilized. Purified cell wall (10 mg) was hydrolyzed in a sealed tube with 6N-distilled HCl (10 ml), at 110°C for 24 h [16]. Glucosamine was estimated in the hydrolysate [17]. Calibration curve was prepared by using 10–100 μg of *N*-acetyl glucosamine. Amino acids were analyzed in the cell wall hydrolysate

in duplicates after precolumn derivatization by using phenylisothiocyanate [18]. The phenyl thiocarbamoyl amino acids were analyzed by Pico-Tag amino acid analysis system [19]. Total sugar of the cell wall was estimated by phenol sulphuric acid method.

Plate assay for antibiotic sensitivity

Plates containing nutrient agar (HiMedia Laboratories Limited, Mumbai, India), IM agar (IM with 1.8% agar) and complex medium with 1.8% agar (composition described above) were lawned with 0.2 ml of culture broth that contained 2×10^4 cfu/ml of *B. flexus*. Antibiotic octodisc (HiMedia Laboratories Limited, Mumbai, India) containing various antibiotics as shown in Table 3 was placed on the agar surface and the plates were incubated at 30°C up to 48 h. Clearance zone surrounding the individual antibiotic was measured in millimeter. Experiment was carried out in triplicate.

Characterization of PHA

^{1}H-Nuclear magnetic resonance spectroscopy (NMR) spectra were recorded using purified PHA (5 mg) in deuterated chloroform at 400 MHz in a AMX 400 (Bruker) spectrophotometer. PHB, P(HB-co-HV)-5 mol% obtained from Sigma-Aldrich, USA, were used as standards for comparison.

Results and discussion

Identification of *Bacillus* species

Amongst various bacteria that were obtained from the composting soil, one of the *Bacillus* species produced 50% PHA in the biomass. The isolate was initially identified as *Bacillus* species because it was gram-positive, catalase positive, motile, endospore forming, rod shaped and aerobic bacterium. The genomic DNA was used for the amplification of 16S rRNA gene by PCR and a comparative search for this sequence revealed 99.4% homology to *B. flexus*. Further, the BLAST search using the sequence revealed that it clustered with other strains such as *Bacillus megaterium* and *B. simplex* (Fig. 1).

Cultivation of *B. flexus* and extraction of PHA

Bacterium cultivated in shake flasks resulted in 2 g/l biomass with 50% PHA (Table 1). The standard strain produced 3.2 g/l of biomass, however the PHA concentration of the cell was very low (3.7%). *B. flexus* was cultivated under optimized conditions in a fermentor wherein 6.8 g/l of biomass and 3.8 g/l of PHA (56% of biomass) were obtained during 24–36 h growth period (Fig. 2).

Complex medium containing peptone, yeast extract and beef extract stimulated growth (3 g/l), and to find out their effect on growth and PHA, they were substituted individually to IM (Table 1). Enhanced growth was obtained in the presence of peptone (4.1 g biomass/l) and yeast extract (3.9 g biomass/l) and poor growth was observed in beef extract medium (1.6 g biomass/l) compared to control inorganic medium (2 g/l). However maximum concentration of PHA in the cells was synthesized in the IM (50%). The results indicated that the cells harvested from inorganic medium and from beef extract medium which had poor growth lysed easily and the recovery of PHA was more in chloroform extraction or mild alkali digestion (86–100%) compared to those grown in organic nutrient medium containing peptone and yeast extract (32–56%). Shape of cells differed under different nutrient provided in the media. It is reported that under limitation in the supply of phosphate, mutant cells of *B. licheniformis* formed irregular spheres, which changed back to rods when phosphate was supplied [20]. This is assigned to the enzyme deficiency, which is linked to cell wall synthesis.

Effect of nutrients on cell wall composition of *B. flexus*

Studies were carried out in duplicate to find out the effect of organic and inorganic nutrients on cell wall compositions of *B. flexus* (Table 2). Concentration of glucosamine was not affected by organic or inorganic nutrients (8.8 mg/100 mg of cell wall). The cell walls contained glutamic acid, alanine, arginine, valine, leucine, tyrosine, aspartic acid and isoleucine. Under peptone and yeast extract supplementation higher concentration of amino acids were observed in the cell walls compared to those cultivated in inorganic salts medium. Cells grown in the presence of peptone and yeast extract contained detectable amounts of diaminopimelic acid (DAP) in their cell walls, which was totally absent in those grown in the inorganic salts medium.

The mucopeptide of *Bacillus* species cell wall contains diaminopimelic acid, glutamic acid, alanine, *N*-acetylglucosamine, *N*-acetylmuramic acid and amide groups [20]. The cell wall also contains *N*-acetylgalactosamine, phosphorous, glycerol and glucose. The cell wall peptide has four amino acids and it is usually made up of alanine and glutamic acid. Different peptidoglycan structures are formed due to substitutions of diamino acids found at the third position of the peptide. The most common diamino acids are lysine and DAP. DAP containing peptide participates in cross bridge

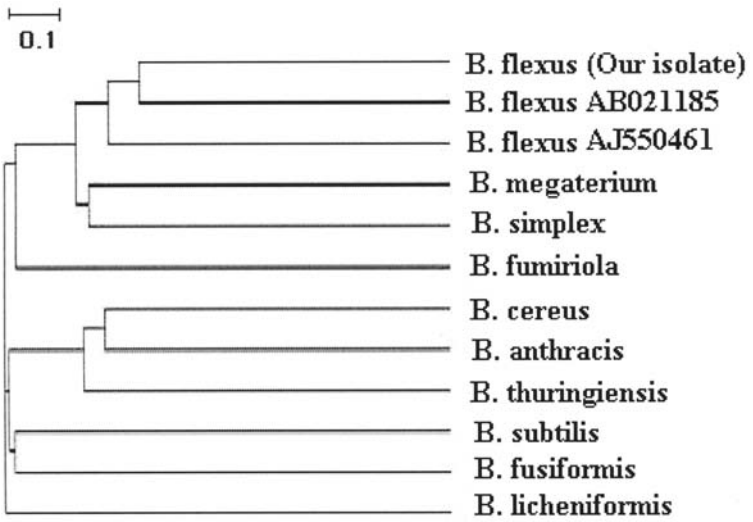

Fig. 1 Phylogenetic tree showing the position of the isolated *B. flexus* compared to other known *Bacillus* species.

Table 1 Production of PHA by isolated culture of *Bacillus flexus* in various media and its extraction

Medium	Cell size (μm) and morphology	Biomass (g/l)	PHA (g/l)	PHA extracted (% of biomass)		
				A	B	C
IM*	1 × 6 ± 0.70 Pleomorphic	2.0 ± 0.84	1.0 ± 0.42	50	43	50
IM[a]	1 × 2 ± 1.41 Small rods	3.9 ± 0.91	1.6 ± 0.28	40	13	13
IM[b]	1 × 3 ± 0.70 Small rods	1.6 ± 0.35	0.7 ± 0.21	44	39	44
IM[c]	1 × 4 ± 0.70 Pleomorphic	4.1 ± 0.28	1.6 ± 0.28	30	17	12
Complex medium	1 × 5 ± 0.70 Pleomorphic	3.0 ± 0.84	1.4 ± 0.42	48	38	23
B. flexus (MTCC 2909) in IM	1 × 6 ± 0.70 Pleomorphic	3.2 ± 0.91	0.1 ± 0.02	3.7	–	–

*Inorganic nutrient medium with $(NH_4)_2SO_4$.
IM[a], IM[b] and IM[c] media are similar to inorganic nutrient medium [without $(NH_4)_2SO_4$], but with supplementation of yeast extract (2.5 g/l); beef extract (2.5 g/l) and peptone (5.0 g/l), respectively.
A = Hypochlorite hydrolysis; B = Chloroform extraction; C = Alkaline hydrolysis.

formation between DAP and alanine or between two DAP residues. Such cross bridge formations provide structural stability to the cell. It is difficult to disrupt the cell walls of gram-positive bacteria such as *Bacillus* species due to the presence of thicker peptidoglycan layer, which is cross-linked. The formation of peptidoglycan is dependent on the uptake of nitrogen and its metabolism to provide the required peptides for peptidoglycan synthesis. Variations are known to occur in the chemical composition of the cell wall of *B. subtilis* during growth in different media [21]. In our study, the presence of DAP in the cell walls of cells grown in peptone and yeast extract containing medium confirmed that the cell wall is more stable compared to those grown in inorganic medium. Cells cultivated in complex medium containing ammonium sulfate as one of the nitrogen sources lacked DAP, had lower concentrations of amino acids in the cell wall, compared to peptone/yeast extract grown cells. This allowed easier extractability of the polymer. The changes may be due to altered metabolic activities in the presence of inorganic nitrogen source.

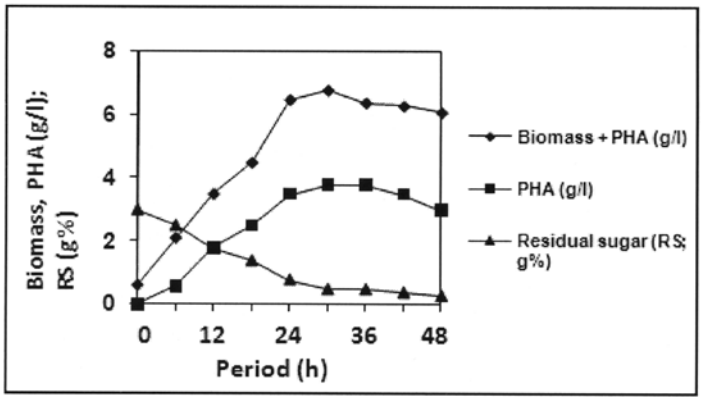

Fig. 2 Fermentor cultivation of *B. flexus*: the bacterium was cultivated in inorganic nutrient medium with sucrose as a carbon source at 30°C, 50% dissolved oxygen and constant pH 7.

Table 2 Amino acid composition of the cell walls isolated from *B. flexus* cultivated in inorganic and organic nutrient media

Amino acids	Amino acid in isolated cell wall (mg/100 mg)				
	1	2	3	4	5
Aspartic acid	0.26	0.95	1.08	0.10	0.52
Glutamic acid	0.62	1.12	1.12	0.18	0.81
Arginine	0.27	0.89	1.05	0.09	0.42
Alanine	0.30	0.75	0.73	0.09	0.42
Tyrosine	0.13	1.00	0.6	0.03	0.07
Valine	0.26	0.95	0.98	0.10	0.48
Isoleusine	0.23	0.81	0.81	0.08	0.30
Leusine	0.33	1.06	1.1	0.13	0.44
Diaminopimelic acid	–	Present	Present	–	–

1 = Inorganic nutrient medium (IM); 2 = IM with yeast extract, 3 = IM with peptone, 4 = IM with beef extract, 5 = Complex medium; Composition of media is given under materials and methods.

Table 3 Antibiotic sensitivity (clearance zone) of *B. flexus* cultivated in various agar plate media for 48 h at 30°C

Antibiotics used (as octodisc)	Clearance zone (in mm) on agar plates		
	Nutrient agar	Inorganic medium*	Organic medium**
Tetracyclin (10 µg)	40 ± 1.41	40 ± 1.41	30 ± 0.70
Sulphamethaxazole (25 µg)	22 ± 2.82	40 ± 1.41	20 ± 0.70
Trimethoprim (125 µg)	22 ± 2.12	22 ± 1.41	28 ± 0.70
Erythromycin (5 µg)	16 ± 0.70	16 ± 0.70	16 ± 0.70
Fusidic acid (10 µg)	12 ± 1.41	24 ± 0.70	12 ± 1.41
Gentamycin (10 µg)	12 ± 1.41	22 ± 0.70	12 ± 0.70
Clindamycin (2 µg)	32 ± 0.00	16 ± 0.70	32 ± 0.70
Penicillin G (1 unit)	24 ± 1.41	24 ± 0.70	24 ± 1.41

*,**Details given under materials and methods in the text.

Cells subjected to organic or inorganic nutrition also showed variations in susceptibility or resistance to certain antibiotics (Table 3). Antibiotics are rendered inactive: (a) through enzymes; (b) altered uptake due to changes in the outer membrane and the channel through which the molecules move into the cell; and (c) altered target site or antibiotic binding proteins [22]. It has been reported that different media would influence the susceptibility of bacteria to certain antibiotics [23]. In the present study changes in the resistance or susceptibility appears to be based on nutritional status of the cell which also leads to altered cell wall nature.

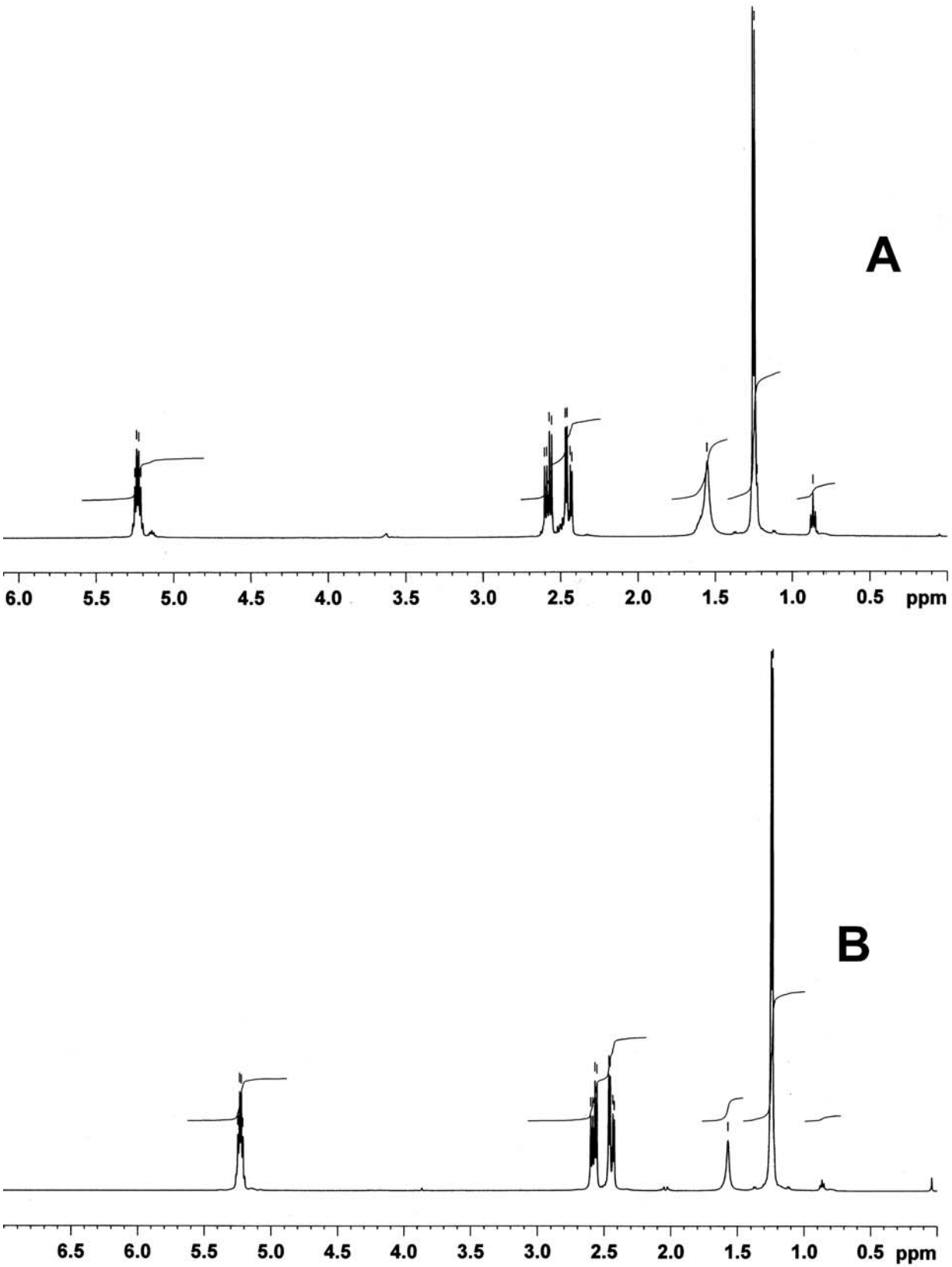

Fig. 3 ^{1}H-NMR spectrum of PHA: (A) Standard P(HB-co-HV); (B) PHA extracted from *B. flexus* cultivated in sucrose containing medium.

Characterization of PHA

GC analysis of PHA isolated from *B. flexus* indicated that it was a copolymer of P(HB-co-HV) of 98:2 mol%. This was confirmed by ^{1}H-NMR (Fig. 3). The spectrum was comparable to the standard P(HB-co-HV) and the reported data [7].

Conclusion

The results demonstrated that the composition of cell wall of *B. flexus* is significantly influenced by the composition of the growth medium. Cells grown in inorganic medium lysed easily and this can be further exploited for easier recovery of the intracellular PHA.

Acknowledgements The authors thank The Department of Biotechnology, Ministry of Science and Technology, Govt. of India for the financial support to the project; Dr. Lalitha R. Gowda and Dr. Prakash M. Halami, for the help rendered in amino acid and 16S rRNA work, respectively; Director, CFTRI, Mysore, India, for the encouragement given to carry out the research program. M. S. Divyashree wishes to thank Council of Scientific and Industrial Research, India, for providing Research Fellowship.

References

1. Choi J and Lee S (1999) Efficient and economical recovery of poly 3 hydroxybutyrate from recombinant *Escherichia coli* by simple digestion with chemicals. Biotechnol Bioeng 62:546–553
2. Doi Y, Kitamura S and Abe H (1995) Microbial synthesis and characterization of polyhydroxybutyrate-co-hydroxyhexanoate. Macromolecules 28:4822–4828
3. Matsusaki H, Abe H and Doi Y (2000) Biosynthesis and properties of polyhydroxybutyrate-co-hydroxyvalerate by recombinant strain of *Pseudomonas* sp 61-3. Biomolecules 1:17–22
4. Berger E, Ramsay JA, Chavarie C and Braunegg G (1989) PHB recovery by hypochlorite digestion of non-PHB biomass. Biotechnol Tech 3:227–232
5. Holmes PA and Lim GB (1990) Separation process, U.S. Patent, 4910145
6. Kshama L and Shamala TR (2006) Extraction of polyhydroxyalkanoate from *Sinorhizobium meliloti* cells using *Microbispora* sp. culture and its enzymes. Enzyme Microb Technol 39:1471–1475
7. Labuzek S and Radecka I (2001) Biosynthesis of PHB tercopolymer by *Bacillus cereus* UW 85. J Appl Microbiol 90:353–357
8. Chen GQ, Konig KH and Lafferty RM (1991) Occurrence of poly β-hydroxyalkanoates in the genus *Bacillus*. FEMS Microbiol Lett 84:173–176
9. Shamala TR, Chandrashekar A, Vijayendra SVN and Kshama L (2003) Identification of polyhydroxyalkanoate (PHA) producing *Bacillus* spp using the polymerase chain reaction (PCR). J Appl Microbiol 94:369–374
10. Sambrook J, Fritch EF and Maniatis T (1989) *Molecular Cloning: A Laboratory Manual,* 2nd edition, Cold Spring Harbor Laboratory Press, New York
11. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
12. Williamson DH and Wilkinson JF (1958) The isolation and estimation of poly-β-hydroxybutyrate inclusions of *Bacillus* sp. J Gen Microbiol 19:198–209
13. Law JH and Slepecky RA (1961) Assay of polyhydroxy-butyric acid. J Bacteriol 82:33–36
14. Brandl H, Gross RA, Lenz RW and Fuller RC (1988) *Pseudomonas oleovorans* as a source of poly-β-(HA) for potential application as biodegradable polyesters. Appl Environ Microbiol 54:1977–1982
15. Schnaitman CA (1981) Cell fractionation. In: *Manual Methods for General Microbiology*. Gerhardt P, Murry RCF (Eds.), American Society for Microbiology Press, Washington, DC, 52 pp
16. Gouda KGM, Lalitha RG, Appu Rao AG and Prakash V (2006) Angio I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (*Glycine max*). J Agri Food Chem 54:4568–4573
17. Good TA and Bessman SP (1964) Determination of glucosamine and galactosamine using borate buffers for modification of the Elson-Morgan and Morgan-Elson reactions. Anal Biochem 9:253–262
18. Bidlingmeyer BA, Cohen SA, Tarvin TL and Frost B (1987) A new rapid high sensitivity analysis of amino acids in food type samples. J Assoc Off Anal Chem 70:241–247
19. Bennett HPJ and Solomon S (1986) Use of pico-tag methodology in the chemical analysis of peptides with carboxyl-terminal amides. J Chromatography 359:221–230
20. Forsberg CW, Wyrick PB, Ward JB and Rogers HJ (1973) Effect of phosphate limitation on the morphology and cell wall composition of *Bacillus licheniformis* and its phosphoglucomutase-deficient mutants. J Bacteriol 113: 969–984
21. Young FE (1965) Variation in the chemical composition of the cell walls of *Bacillus subtilis* during growth in different media. Nature 207:104–105
22. Neu HC (1992) The crisis in antibiotic resistance. Science 257:1064–1073
23. Huys G, Haene KD and Swings J (2001) Influence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method. Lett Appl Microbiol 34:402–406