Indian J Microbiol (September 2009) 49:251–258
DOI: 10.1007/s12088-009-0031-z

ORIGINAL ARTICLE

Production and characterization of bacterial polyhydroxyalkanoate copolymers and evaluation of their blends by fourier transform infrared spectroscopy and scanning electron microscopy

T. R. Shamala · M. S. Divyashree · Reeta Davis · K. S. Latha Kumari · S. V. N. Vijayendra · Baldev Raj

Received: 20 February 2008 / Accepted: 23 July 2008

Abstract *Rhizobium meliloti* produced a copolymer of short chain length polyhydroxyalkanoate (scl-PHA) on sucrose and rice bran oil as carbon substrates. Recombinant *Escherichia coli* (JC7623ABC1J4), bearing PHA synthesis genes, was used to synthesize short chain length-co-medium chain length PHA (scl-co-mcl-PHA) on glucose and decanoic acid. Fourier transform infrared spectroscopy (FTIR) spectra of the PHAs indicated strong characteristic bands at 1282, 1723, and 2934 cm^{-1} for scl-PHA and at 2933 and 2976 cm^{-1} for scl-co-mcl-PHA polymer. Differentiation of polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-hydroxyvalerate-P(HB-co-HV) copolymer was obseverd using FTIR, with absorption bands at 1723 and 1281 for PHB, and at 1738, 1134, 1215 cm^{-1} for HV-copolymer. The copolymers were analyzed by GC and ^{1}H NMR spectroscopy. Films of polymer blends of PHA produced by *R. meliloti* and recombinant *E. coli* were prepared using glycerol, polyethylene glycol, polyvinyl acetate, individually (1:1 ratio), to modify the mechanical properties of the films and these films were evaluated by FTIR and scanning electron microscopy.

T. R. Shamala[1] · M. S. Divyashree[1] · R. Davis[2] · K. S. L. Kumari[1] · S. V. N. Vijayendra[1] (✉) · B. Raj[3]
[1]Department of Food Microbiology,
[2]Department of Plant Cell Biotechnology,
[3]Department of Food Packaging Technology,
Central Food Technological Research Institute,
Mysore - 570 020, India

E-mail: svnvijayendra@yahoo.com

Keywords Polyhydroxyalkanoate · Fourier transform infrared spectroscopy · PHA blends · *Rhizobium meliloti* · Recombinant *Escherichia coli* · Scanning electron microscopy

Introduction

Several bacteria produce polyhydroxyalkanoates (PHAs) under nutrient depletion conditions. PHAs are divided into two classes: short chain length PHA (scl-PHA) that has 3-5 carbon atoms and medium chain length PHA (mcl-PHA) that consists of 6–14 carbon atoms. Polyhydroxybutyrate (PHB), an scl-PHA, is found in many bacterial cells and it is known to be a brittle thermoplastic. The nature and proportion of different monomers are influenced by the bacterial strains, type and relative quantity and quality of carbon sources supplied to the growth medium [1]. Increase in hydroxyvalerate (HV) content in the copolymer containing poly(hydroxybutyrate-co-hydroxyvalerate)-P(HB-co-HV) leads to improved mechanical property such as flexibility and strength [2]. However supplementation of fatty acids to the growth medium for copolymer production affects the economics of polymer production [3]. Attempts have been made to decrease the brittleness of PHB by incorporating co-monomer such as hydroxyvalerate, or by blending PHB with other polymers [4, 5]. Plasticizers such as laprol, dibutylsebacate, dioctylsebacate, polyisobutylene and polyethylene glycol have been used to alter the mechanical properties and biodegradation character of PHA from *Azotobacter* sp. [6]. For such assessment, the films are prepared by solution casting technique and then analyzed,

which requires sizable quantity of polymer. Fourier transform infrared spectroscopy (FTIR) has been used as a versatile and noninvasive analytical tool for the qualitative studies of PHA in bacterial cells [7]. The intense absorption bands around 1724 cm^{-1} represents the stretching vibration of C=O groups, which is regarded as the characteristic band for PHA. Other accompanying bands located near 1280 and 1165 are attributed to C-O-C groups. The CH stretching bonds in the polyester is assigned to the bands located in the spectral region around 2900 cm^{-1}. In the present work, scl-PHA and scl-co-mcl-PHA copolymers were produced using *R. meliloti* and recombinant *E. coli* strains, the polymers obtained were blended with glycerol, polyethylene glycol and polyvinyl acetate to modify the mechanical properties and the samples were characterized using FTIR.

Materials and methods

PHB and P(HB-co-HV) were obtained from Sigma Aldrich (USA). Plasticizers such as polyethylene glycol 400 (PEG), glycerol and polyvinyl acetate (PVAc) were procured from Hi-media Laboratories, Mumbai, India.

Microorganisms and their maintenance

Rhizobium meliloti isolated from the root nodules of *Trigonella foenum graecum* was identified [8] and maintained on yeast mannitol agar slants (Hi-media Laboratories, Mumbai, India) at 4°C. Recombinant *E. coli* (JC7623ABC1J4) harboring *PhaA* (β-ketothiolase) and *PhaB* (acetoacetyl CoA reductase) genes from *Bacillus* sp. and *PhaC1* (PHA synthase) and *PhaJ4* (R-specific enoyl CoA hydratase) genes from *Pseudomonas aeruginosa*, were cloned to construct a recombinant *Escherichia coli* strain (JC7623ABC1J4). This was constructed in the lab (data for which is not shown here). It was used to produce scl-co-mcl-PHA for comparison. The culture was maintained on Luria Bertani (LB) agar (Hi-media Laboratories, Mumbai, India) slants at 4°C.

PHA production from *R. meliloti*

To prepare inoculum, actively growing cells of *R. meliloti* were transferred into 500 ml capacity Erlenmeyer flasks containing 100 ml sterile inoculum medium (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, 10; yeast extract, 0.5 (pH-7.0). The inoculated flasks were incubated on a shaker at 30°C, 250 rpm for 24 h.

PHA production medium (100 ml in 500 ml capacity Erlenmeyer flask), which was similar to the above-mentioned inoculum medium but devoid of yeast extract and containing higher concentration of sucrose of 20 g/l, was inoculated with 10% (v/v) of the inoculum (1.8 x 10^4 cfu/ml), in triplicate and flasks were incubated at 250 rpm, 30°C for 72 h. To enhance P(HB-co-HV) copolymer production, 200 mg% (w/v) of sterile rice (*Oryza sativa*) bran oil (a co-substrate), suspended in 5 ml of water, was added to each flask after 24 h of fermentation. Rice bran oil procured from local market mainly contained palmitic acid (15%), oleic acid (43%) and linoleic acid (39%).

PHA production from recombinant *E. coli*

Cells of recombinant *E. coli* harboring bacterial PHA synthesis genes were transferred to 10 ml LB broth (LB, Himedia, Mumbai, India) contained in test tube. Ampicillin sodium salt (100 mg/l) was added to the inoculated medium for sustenance of the recombinant plasmid and the tubes were incubated at 37°C at 200 rpm for 8 h. This inoculum (2×10^3 cfu/ml) was added to 100 ml LB medium that additionally contained (g/l) glucose, 20; and filter-sterilized decanoic acid, 2; as main and co-carbon sources, respectively. Incubation (200 rpm) was carried out for 48 h at 37°C. Isopropyl-β-D-thiogalacto pyranoside (IPTG) was added (0.4 mmol) as an inducer after 18 h of growth period.

Estimation of biomass and PHA content

Cells were harvested by centrifugation at 8000 rpm for 15 min, washed with distilled water and dried at 50°C to a constant weight. PHA from bacterial cells was isolated and purified by solvent extraction method [9]. Qualitative and quantitative estimation of PHA was carried out by GC analysis using lyophilized cells [10]. The lyophilized cells (10 mg) were subjected to methanolysis at 100°C for 140 min in 1 ml chloroform, 0.85 ml methanol and 0.15 ml concentrated H_2SO_4. The samples were homogenized with deionized water and the bottom organic phase was used for GC analysis. Analysis was carried out using a 30 m DB-1(fused silica gel-polymethyl siloxane) capillary column (internal diameter 0.25 mm and film thickness 0.25 microns). Carrier gas used was N_2 (1 ml /min). The injector and the detector temperatures were maintained at 170°C and 220°C, respectively. The temperature program used was: 55°C for 7 min; ramp of 4°C per min up to 100°C; 10°C per min rise up to 200°C and hold at 200°C for 10 min. PHB, P(HB-co-HV), containing 5 mol% of hydroxyvalerate (Sigma Aldrich, USA) was used as the standard. Benzoic acid was used as the internal standard.

FTIR and ^{1}H NMR spectroscopy

Standard and sample PHA (5 mg from *R. meliloti* and recombinant *E. coli*) were mixed with 100 mg of FTIR

grade KBr and pelletized. The FTIR spectrum was recorded at 400-4000 cm^{-1} in FTIR Nicolet Magna 5700 spectro-photometer (Thermo electron Inc., NICOLET 5700, FT – Raman Module, USA). Alternately, PHA samples (10 mg) were dissolved in 200 µl of chloroform and placed in KBr window and spectrum was recorded. PHA from *R. meliloti* and recombinant *E. coli* (5 mg each) were mixed with plasticizers (5 mg each) namely glycerol, PVAc and PEG in 1:1 ratio and pelletized with KBr/solubilized in chloroform and FTIR spectra were recorded as mentioned above. For NMR analysis, 5 mg PHA samples were dissolved in $CDCl_3$ (1 ml) and analyzed at 400 MHz in AMX 400 (Bruker) NMR spectrophotometer (Germany).

Properties of the PHA films

PHA blends were prepared by homogenizing 370 mg each of PHA with PEG, glycerol or PVAc in 40 ml of chloroform and the solutions were poured onto 10 × 5 cm, leveled glass plate and dried at 30°C to obtain the films. PHA filmstrips (10 × 1 cm) of known thickness were used for mechanical tests. Tensile strength and % elongation were determined using a universal texture measuring system (LLOYD instruments LR5K, UK) at 30°C using 50 N load cell with a speed of 50 mm/ min. PHA films and the blends were gold sputter coated and analyzed in a scanning electron microscope (LEO-435, Cambridge, UK).

Determination of molecular weight of PHA

Molecular weight (Mw) of PHA samples from *R. meliloti* and recombinant *E. coli* and their blends with PVAc were determined in chloroform solution of the test material (0.1-2.0% w/v), by viscosity method using Oswald viscometer at 20°C. Graph was plotted for reduced viscosity vs. concentration, to find the intrinsic viscosity. Mw was determined using the formula [11]: Intrinsic viscosity $=K \times (Mw)^{0.82}$, where, K-constant is 7.7×10^{-5}. PHB, P(HB-co-HV) of 5 mol% was used as the standard.

Results and discussion

Production of PHA copolymer

PHB, which is generally found as energy reserve of bacterial cells is brittle and copolymerization with other alkanoates leads to improved mechanical property such as flexibility and strength [2, 12]. *R. meliloti* produced about 60% PHA of the biomass with sucrose as the main carbon source. The polymer contained P(HB-co-HV) of 97:3 mol% (Table 1). HV concentration increased to 5% by co-feeding of rice bran oil during cultivation. Recombinant *E. coli* produced 21% of scl-co-mcl PHA (71:29) copolymer with glucose and decanoic acid as carbon sources. Polymer was composed of butyrate, hexanoate and octanoate in 71:8:21 mol%.

Detection of PHA copolymer

FTIR is one of the rapid and powerful tools to obtain information on polymer structure, because every chemical compound in the sample makes its own distinct contribution to the absorbance/transmittance spectrum. The method is particularly suitable for screening a large number of bacterial cells and it has been demonstrated that PHA present within the cells can be rapidly detected by this technique [7]. In the present study, it was observed that FTIR spectra of PHAs containing short chain length monomers such as HB and HV) and medium chain length hydroxyalkanoate monomer (hydroxyhexanoate-HHx; hydroxyoctanoate HOc) pelletized by KBr showed variations in band patterns (Fig. 1). The transmittance bands located at 1724–1740 cm^{-1} are attributed to the stretching vibration of the C=O group (ester carbonyl) in the PHA polyester. Accompanying bands of the C-O-C groups appear in the spectral region from 1150 to 1300 cm^{-1}. Transmittance region from 2800–3100 cm^{-1} corresponds to stretching vibration of C-H bonds of methyl (CH_3), and methylene (CH_2) groups. Other characteristic bands present for scl PHA were 2977, 2934, 1282 (CH_3 bend), 1100, 1058 (C-O), 979 and 515. Scl-co-mcl PHA had the strongest methylene

Table 1 Synthesis of PHA by *R. meliloti* and recombinant *E. coli* and their composition

Bacteria	Carbon source	Biomass (mg%)	PHA (% of biomass)	Mol%* of PHA components HB : HV : HHx : HOc			
R. meliloti	Sucrose	300±28**	62±5	97	3	0	0
R. meliloti	Sucrose + rice bran oil	315±7	60±5	95	5	0	0
Recombinant *E. coli*	Glucose + decanoic acid	120±14	21±3	71	0	8	21

*By GC analysis; **Average values of three experiments with SD; HB = hydroxybutyrate; HV = hydroxyvalerate; HHx = hydroxyhexanoate; HOc = hydroxyoctanoate

–C-H- vibrations near 2933 cm^{-1}. PHB standard and PHA from *R. meliloti* also showed bands similar to that of scl-PHA. The results indicated that by using KBr pelletized PHA, it was possible to distinguish scl from mcl PHA, but within scl-PHA, HV could not be conclusively distinguished from HB.

Minor band differences in the absorption spectrum of homopolymer of PHB and co polymers of P(HB-co-HV) in chloroform as a medium were noticed. PHB homopolymer had characteristic absorption bands of 1724 and 1280, while copolymer of P(HB-co-HV) showed a shift in carbonyl band to 1737 and showed additional peaks at 1303, 1229, 1196 and 797 cm^{-1}. *R. meliloti* PHA, which was also a copolymer of P(HB-co-HV) showed similar bands (data not shown). Recombinant *E. coli*, which produced scl-co-mcl PHA, showed characteristic bands at 1739, 1262, 778, 756, 747 and 737 cm^{-1}. These variations may be due to differences in the physical state of the PHA in chloroform. FTIR spectroscopy has also been used to investigate and even predict the degree of crystallinity of PHB samples [13]. Production of P(HB-co-HV) and scl-co-mcl PHA by *R. meliloti* and recombinant *E. coli,* respectively, were confirmed by ^{1}H NMR spectroscopy. ^{1}H NMR spectrum of PHA showed signals characteristic of PHB: a doublet at 1.29 ppm, which is attributed to the methyl group, a doublet of quadruplet at 2.5 ppm, which is attributed to methylene group and a multiplet at 5.28 ppm characteristic of methyne group. A triplet at 0.9 ppm and a methylene resonance at 1.59 and methyne resonance at 5.15 indicated the presence of valerate in the polymer. PHA from recombinant *E. coli* showed resonance of PHB and additional resonance of mcl-PHA (Fig. 2). The NMR spectrum obtained is in accordance with data reported in the literature [14, 15].

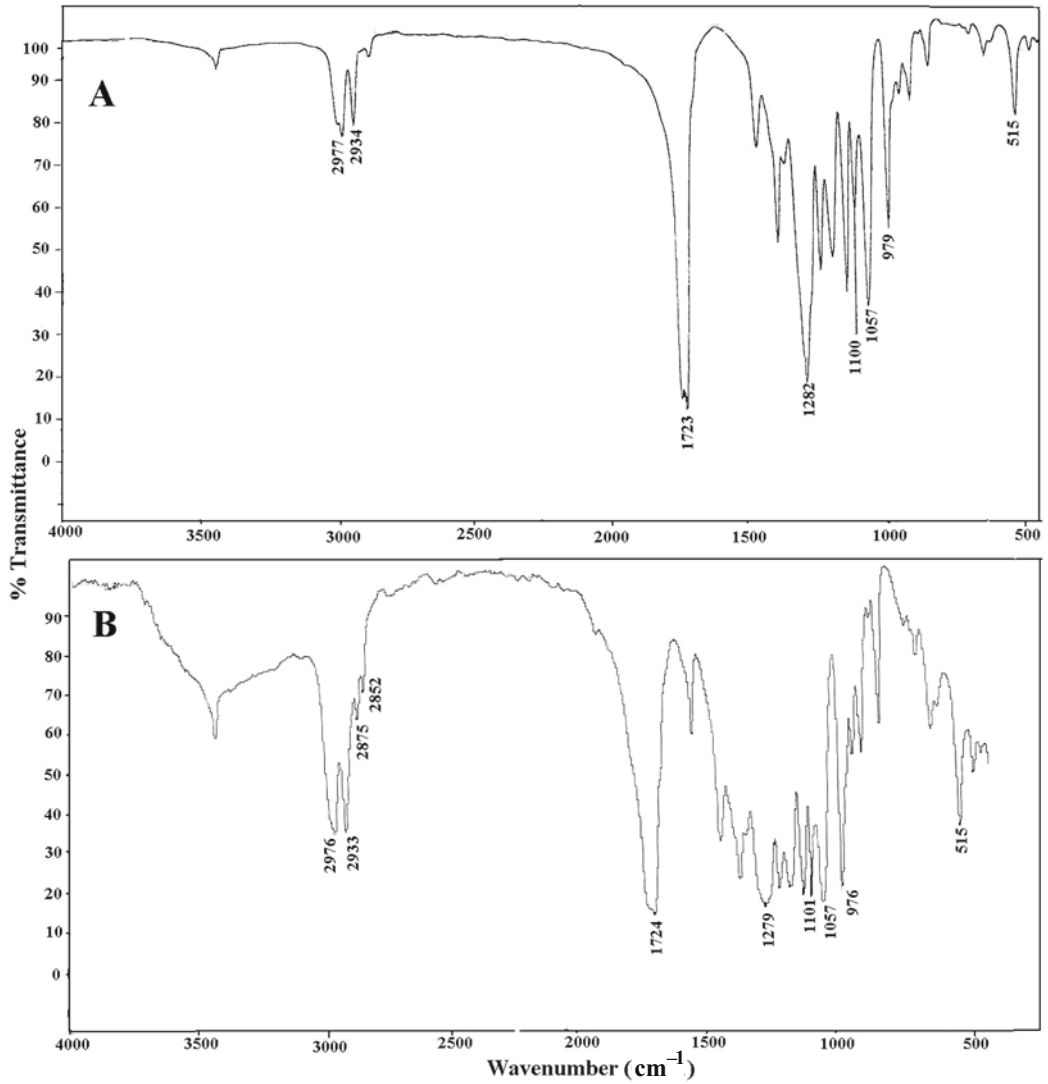

Fig. 1 FTIR spectrum of KBr pelletized PHA: (A) Standard scl-PHA (PHB); (B) scl-co-mcl PHA of recombinant *E. coli*

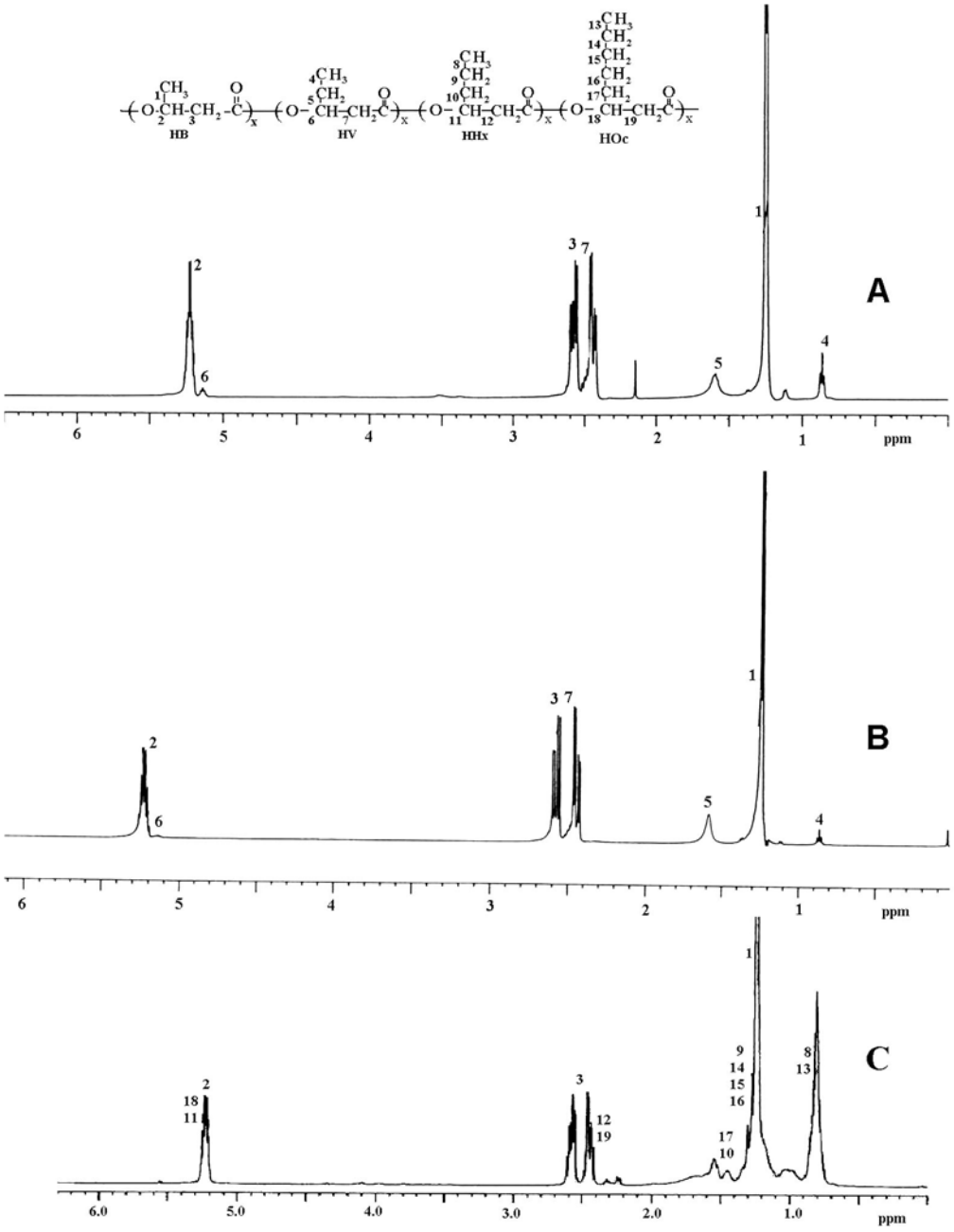

Fig. 2 ^{1}H NMR spectrum of A) Standard P(HB-co-HV); B) PHA from *R. meliloti* C) PHA from recombinant *E. coli;* indicating HB = hydroxybutyrate; HV = hydroxyvalerate; HHx = hydroxyhexanoate and HOc = hydroxyoctanoate

Blending of the PHA polymers

PHB alone is not used for various applications due to its brittleness. It does not contain any functional group for chemical modification other than the hydroxyl and carboxylic groups at chain ends. The strategies to enhance mechanical property involve production of PHA copolymers by fermentative methods or making miscible blends of PHA with other polymers or plasticizers. PHA has been blended earlier with chitosan, starch, laprol, PEG etc. to produce film of better mechanical property [5, 16, 17].

In the present study, PHA obtained from *R. meliloti*, which contained 3 mol% of HV and PHA from recombinant *E. coli*, which contained scl-co-mcl PHA (71:29 mol%) were used for blending. Standard PHB was also used for comparison. Analysis of blends by FTIR using KBr pelletization indicated that the PHB was less reactive with glycerol, PEG and polyvinyl acetate (Table 2). PHA from

R. meliloti and recombinant *E. coli* were more reactive with polyvinyl acetate than with glycerol or PEG. Characteristic vibration bands for PVAc alone were noticed at 1735 cm^{-1} for carbonyl stretching and at 1249 cm^{-1} for C-O stretching. Major band shift was noticed in C=O regions for the blends. Suppression of crystallinity is assigned to the formation of intermolecular hydrogen bonds between PHA carbonyls and the blend substrate used [17]. This is also evidenced by the FTIR band intensity of the carbonyl stretching absorption from PHA and its blends using PVAc as a plasticizer (Tables 2 and 3). The lowest band (1724 cm^{-1}) is assigned due to crystalline phase and stronger absorption bands at 1735, 1743 cm^{-1} are attributed to amorphous phase [17]. *E. coli* + PVAc and PVAc alone was 6.7×10^4, 1.1×10^5, 8.6×10^4, 9.4×10^4 and 4.4×10^4, respectively, which indicated modification of the blended material that would lead to altered mechanical properties.

Scanning electron microscopy of the PHA films

A study on the morphology of the blends was carried out using scanning electron microscopy (SEM) and it was observed that PHB exhibited a surface with high porosity. Non-reactivity of PHB with PEG/glycerol/PVAc was observed as granulated/porous surfaces (Fig. 3). Blend of *R. meliloti* PHA with PVAc showed a homogenous back-

Table 2 Major FTIR wave number (cm^{-1}) band shifts of PHA blends measured after KBr pelletization

Control			Glycerol blend*			PEG blend*			PVAc blend*		
1	2	3	1	2	3	1	2	3	1	2	3
3436	3437	3437	**3382**	3662	**3382**	**3434**	**3484**	**3409**	3439	3440	3437
2977	2975	2956	Nil	3285	2931	Nil	Nil	**2888**	**2924**	**2956**	**2975**
2934	2933	2926	2937	2941	2881	2875	**2981**	2704	Nil	**2921**	**2933**
1724	1724	1724	1722	1728	1723	1723	**1726**	**1726**	1720	**1735**	**1743**
1282	1280	1280	1311	1284	1279	1280	1283	-	1374	**1240**	**1243**
1132	1132	1132	1109	Nil	Nil	1183	1183	**1113**	Nil	1131	1132
1058	1057	1057	**1044**	1049	**1049**	**1101**	**1101**	1044	1027	1055	1055

1 = Standard PHB; 2 = PHA from *R. meliloti*; 3 = PHA from recombinant *E. coli.* *PHA was blended with respective plasticizers in 1:1 ratio. Bold numbers indicate strong absorption bands compared to respective controls.

Table 3 Major FTIR wave number (cm^{-1}) band shifts of PHA blends measured using chloroform

Control			Glycerol blend*			PEG blend*			PVAc blend*		
1	2	3	1	2	3	1	2	3	1	2	3
3435	3338	3621	3434	3621	3382	3379	3380	**3381**	3435	3690	3449
3008	3020	3020	3009	3020	2931	3004	3008	3008	3019	3021	3020
2932	2980	2978	2932	2986	2881	2912	2912	2912	2928	2977	2923
1723	1738	1738	1723	1738	1723	1724	1738	1738	**1732**	**1735**	**1743**
1281	1215	1215	1281	1215	1279	1290	1215	1247	**1250**	**1251**	**1247**
1101	1134	1134	1101	1134	Nil	1101	1106	1104	1101	1101	1101
1057	1058	1057	1057	1058	1049	-	-	-	1057	1056	1054

1 = Standard PHB; 2 = PHA from *R. meliloti*; 3 = PHA from recombinant *E. coli.* *PHA was blended with respective plasticizers in 1:1 ratio. Bold numbers indicate strong absorption bands compared to respective controls.

This banding pattern was clear in chloroform system than in KBr pellet method.

Pure PHB or its blends could not be made into films. PHA blend with PVAc (1:1) improved the elongation to break % (180%) compared to control film (14%, Table 4). However, there was a decrease in the tensile from 28 Mpa (control) to 7 Mpa of blend film. A balance may be obtained by blending known quantities of PHA and PVAc to attain required tensile strength and % elongation of the film.

Molecular weight of PHA obtained from *R. meliloti*, *R. meliloti* + PVAc blend; recombinant *E. coli*, recombinant

Table 4 Mechanical properties of P(HB-co-HV) of *R. meliloti* blended with glycerol, polyethylene glycol (PEG) and polyvinyl acetate (PVAc)

Composition (1:1)	Tensile strength (Mpa)	% Elongation
P(HB-co-HV)- control	28	14
P(HB-co-HV):Glycerol	4.3	18
P(HB-co-HV):PEG	1.6	50
P(HB-co-HV):PVAc	7.1	180

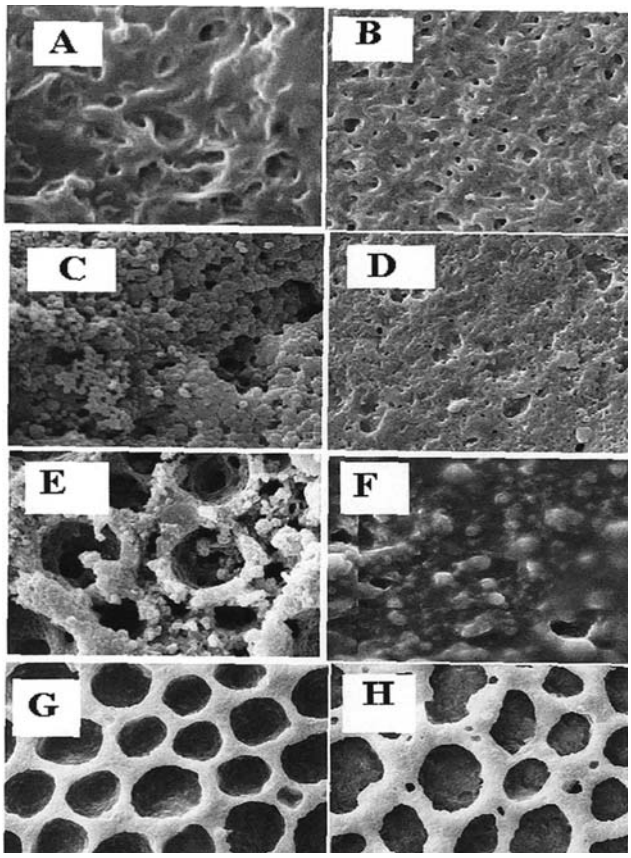

Fig. 3 Scanning electron micrographs showing morphology of control film and blends (Magnification 1.00 K X): A = Control PHB; B = PHA from *R. meliloti*; C = PHB + glycerol; D = PHA from *R. meliloti* + glycerol; E = PHB + PEG; F = PHA from *R. meliloti* + PEG; G= PHB + PVAc; H = PHA from *R. meliloti* + PVAc

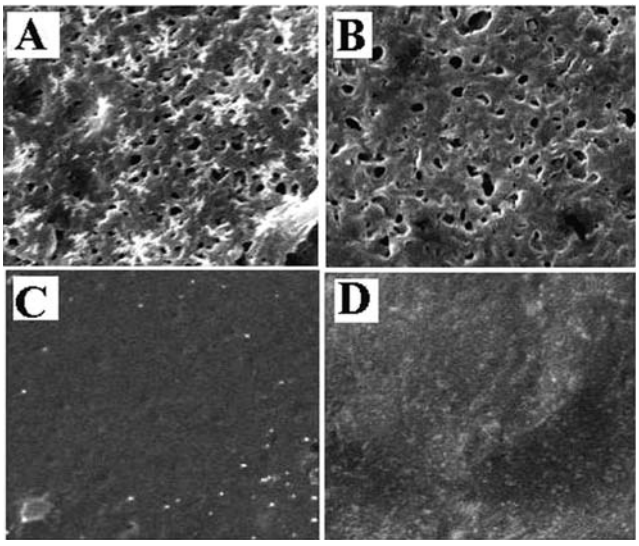

Fig. 4 Scanning electron micrographs showing morphology of recombinant *E. coli* PHA film and its blends (Magnification 1.00 K X): A = Control PHA from recombinant *E. coli*; B = PHA + glycerol; C = PHA + PEG; D = PHA + PVAc

ground layer overlaid with honeycomb-like structure and each cell was thick layered compared to PHB blend. This indicated enhanced reactivity of P(HB-co-HV) compared to PHB. PEG blending appeared as coated surface and glycerol showed minimal blending compared to control (Fig. 3D and 3F). Blend of recombinant *E. coli* PHA with PEG and PVAc also showed better blending and filming characters compared to glycerol (Fig. 4).

Overall results indicated that FTIR could be adopted as a nondestructive method for initial assessment of PHA and its blend quality involving minimal preparative steps for analysis with less amount of sample. Improved mechanical properties may be obtained by using blend formulations of PHA with PVAc.

Acknowledgments The authors thank The Department of Biotechnology, Ministry of Science and Technology, Govt. of India for the financial support to the project, and the encouragement given by the Director, Central Food Technological Research Institute, Mysore, India, to carry out the research program. M. S. Divyashree, Reeta Davis and K. S. Latha Kumari wish to thank the Council of Scientific and Industrial Research, India, for providing Research Fellowships.

References

1. Steinbüchel A, Debzi EM, Marchessault RH and Timm A (1993) Synthesis and production of poly(3-hydroxyvaleric acid) homopolyester by *Chromobacterium violaceum.* Appl Microbiol Biotechnol 39:443–449
2. Lee SY (1996) Bacterial polyhydroxyalkanoates. Biotechnol Bioeng 49:1–14
3. Choi J and Lee SY (2000) Economic considerations in the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by bacterial fermentation. Appl Microbiol Biotechnol 53:646–649
4. Du G, Si Y and Yu J (2001) Inhibitory effect of medium-chain-length fatty acids on synthesis of polyhydroxyalkanoates from volatile fatty acids by *Ralstonia eutropha.* Biotechnol Lett 23:1613–1617
5. Bhatt R, Shah D, Patel KC and Trivedi U (2008) PHA-rubber blends: synthesis, characterization and degradation. Bioresour Technol 99:4615–4620
6. Savenkova L, Gercberga Z, Nikolaeva V, Dzene A, Bibers I and Kalnin M (2000) Mechanical properties and biodegradation of PHA based films. Process Biochem 35: 573–579
7. Hong K, Sun S, Tian W, Chen GQ and Huang W (1999) A rapid method for detecting bacterial PHA in intact cells by FT-IR. Appl Microbiol Biotechnol 51:523–526
8. Vincent JM (1968) The identification and classification of *Rhizobium*. In: Gibbs BM and Shapton DA (eds) Identification methods for microbiologists, Academic Press, London

9. Kshama L and Shamala TR (2003) Enhanced biosynthesis of polyhydroxyalkanoates (PHA) in a mutant strain of *Rhizobium meliloti.* Biotechnol Lett 25:115–119
10. Brandl H, Gross RA, Lenz RW and Fuller RC (1988) *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl Environ Microbiol 54:1977–1982
11. Quagliano JC, Amarila FF, Mata EG and Miyazaki D (2001) Effect of simple and complex carbon sources, low temperature culture and complex carbon feeding polices on poly-3-hydroxybutyric acid (PHB) content and molecular weight (mw) from *Azotobacter chroococcum 6B*. World J Microbiol Biotechnol 17:9–14
12. Amirul AA, Yahya ARM, Sudesh K, Azizan MNM and Majid MIA (2008) Biosynthesis of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) copolymer by *Cupriavidus* sp. USMAA1020 isolated from Lake Kulim, Malaysia. Bioresour Technol 99:4903–4909
13. Bloembergen S, Holden, DA, Hamer GH, Bluhm TL and Marchessault RH **(**1986) Studies of composition and crystallinity of bacterial poly(β-hydroxybutyrate-co-β-hydroxyvalerate). Macromolecules 19:2865–2871
14. Labuzek S and Radecka I (2001) Biosynthesis of PHB tercopolymer by *Bacillus cereus.* UW85. J Appl Microbiol 90: 353–357
15. Pedro AA (2003) Towards new biocompatible materials: From biological polyesters to synthetic copoly (ester-urethanes) for biomedical applications. Dissertation submitted to Swiss federal Institute of Technology, Zurich, pp 46
16. Li X, Liliu K, Jun L, Tan EP, Chan LM, Lim CT and Goh ST (2006) Synthesis, characterization and morphology studies of biodegradable amphiphilic poly [(R)-3-hydroxybutyrate]-alt-poly(ethylene glycol) mulitblock copolymers. Biomacromolecules 7:3112–3119
17. Ikejima T, Yagi Y and InoueY (1999) Thermal properties and crystallization behavior of poly(3- hydroxybutyric acid) in blends with chitin and chitosan. Macromol Chem Phys 200: 413–421