1	Production and characterization of bacterial polyhydroxyalkanoate copolymers and
2	evaluation of their blends by fourier transform infrared spectroscopy and scanning
3	electron microscopy
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1	ABSTRACT Rhizobium meliloti produced a copolymer of short chain length
2	polyhydroxyalkanoate (scl-PHA) on sucrose and rice bran oil as carbon substrates.
3	Recombinant Escherichia coli (JC7623ABC1J4), bearing PHA synthesis genes was used
4	to synthesize short chain length-co-medium chain length PHA (scl-co-mcl-PHA) on
5	glucose and decanoic acid. Fourier transform infrared spectroscopy (FTIR) spectra of the
6	PHAs indicated strong characteristic bands at 1282, 1723, and 2934 cm^{-1} for scl-PHA
7	and at 2933 and 2976 cm^{-1} for scl-co-mcl-PHA polymer. Differentiation of
8	polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-hydroxyvalerate -P(HB-co-HV)
9	copolymer was obseverd using FTIR, with absorption bands at 1723 and 1281 for PHB,
10	and at 1738, 1134, 1215 cm^{-1} for HV-copolymer. The copolymers were analyzed by GC
11	and ¹ H NMR spectroscopy. Films of polymer blends of PHA produced by <i>R. meliloti</i> and
12	recombinant E. coli were prepared using glycerol, polyethylene glycol, polyvinyl acetate
13	individually (1:1 ratio), to modify the mechanical properties of the films and these films
14	were evaluated by FTIR and scanning electron microscopy.
15	
16 17 18 19	Keywords Polyhydroxyalkanoate · fourier transform infrared spectroscopy · PHA blends · <i>Rhizobium</i> <i>meliloti</i> · recombinant <i>Escherichia coli</i> · scanning electron microscopy
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1 INTRODUCTION

23

2 Several bacteria produce polyhydroxyalkanoates (PHAs) under nutrient depletion 3 conditions. PHAs are divided into two classes: short chain length PHA (scl-PHA) that has 4 3-5 carbon atoms and medium chain length PHA (mcl-PHA) that consists of 6-14 carbon 5 atoms. Polyhydroxybutyrate (PHB), which is an scl-PHA is found in many bacterial cells 6 and it is known to be a brittle thermoplastic. The nature and proportion of different 7 monomers are influenced by the bacterial strains, type and relative quantity and quality of carbon sources supplied to the growth medium¹. Increase in hydroxyvalerate (HV) 8 9 content in the copolymer containing poly(hydroxybutyrate-co-hydroxyvalerate)-P(HBco-HV) leads to improved mechanical property such as flexibility and strength². However 10 11 supplementation of fatty acids to the growth medium for copolymer production affects the economics of polymer production³. Attempts have been made to decrease the 12 13 brittleness of PHB by incorporating co-monomer such as hydroxyvalerate, or by blending PHB with other polymers⁴⁻⁵. Plasticizers such as laprol, dibutylsebacate, dioctylsebacate, 14 15 polyisobutylene and polyethylene glycol have been used to alter the mechanical properties and biodegradation character of PHA from Azotobacter sp^6 . For such 16 17 assessment, the films are prepared by solution casting technique and then analyzed, 18 which requires sizable quantity of polymer. Fourier transform infrared spectroscopy 19 (FTIR) has been used as a versatile and noninvasive analytical tool for the qualitative studies of PHA in the bacterial cells⁷. The intense absorption bands around 1724 cm⁻¹ 20 21 represents the stretching vibration of C=O groups, which is regarded as the characteristic 22 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-mclPHA 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.

5

6 Materials and Methods

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

10

11 Microorganisms and their maintenance

12 Rhizobium meliloti isolated from the root nodules of Trigonella foenum graecum was identified⁸ and maintained on yeast mannitol agar slants (Hi-media, Mumbai, India) at 13 14 4°C. Recombinant E. coli (JC7623ABC1J4) [harboring (PhaA (β-ketothiolase) and PhaB (acetoacetyl CoA reductase) genes from Bacillus sp and PhaC1 (PHA synthase) and 15 16 PhaJ4 (R-specific enoyl CoA hydratase) genes from Pseudomonas aeruginosa, were 17 cloned to construct a recombinant Escherichia coli strain (JC7623ABC1J4). This was 18 constructed in the lab, data for which is not shown here], was used to produce scl-co-mcl-19 PHA for comparison. The culture was maintained on Luria Bertani (LB) agar (Hi-media, 20 Mumbai, India) slants at 4°C.

21

22

1 PHA production from *R. meliloti*

2 To prepare inoculum, actively growing cells of *R. meliloti* were transferred into 500 ml 3 capacity Erlenmeyer flasks containing 100 ml sterile inoculum medium (g/l): Na_2HPO_4 4 ·2H₂O, 2.2; KH₂PO₄ 1.5; (NH₄) ₂SO₄ 1.5; MgSO₄·7H₂O, 0.2; sucrose, 10; yeast extract, 5 0.5 (pH-7.0). The inoculated flasks were incubated on a shaker at 30°C, 250 rpm for 24 h. 6 PHA production medium (100 ml in 500 ml capacity Erlenmeyer flask), which was 7 similar to above mentioned inoculum medium but devoid of yeast extract and containing 8 higher concentration of sucrose of 20 g/l, was inoculated with 10% (v/v) of the inoculum (1.8 x 10⁴ cfu/ml), in triplicate and flasks were incubated at 250 rpm, 30°C for 72 h. To 9 10 enhance P(HB-co-HV) copolymer production, 200 mg% (w/v) of sterile rice (Oryza 11 sativa) bran oil (a co-substrate) suspended in 5 ml of water, was added to each flask after 12 24 h of fermentation. Rice bran oil procured from local market mainly contained (%): 13 palmitic acid, 15; oleic acid, 43; and linoleic acid, 39.

14

15 PHA production from recombinant *E. coli*

16 Cells of recombinant *E. coli* harboring bacterial pha synthesis genes were transferred to 17 10 ml Luria Bertani broth (LB, Himedia, Mumbai, India) contained in test tube. 18 Ampicillin sodium salt (100 mg/l) was added to the inoculated medium for sustenance of 19 the recombinant plasmid and the tubes were incubated at 37°C at 200 rpm for 8 h. This 20 inoculum (2 x 10^3 cfu/ml) was added to 100 ml LB medium that additionally contained 21 (g/l) glucose, 20; and filter sterilized decanoic acid, 2; as main and co-carbon sources, 22 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 hrs of growth
 period.

3

4 Estimation of biomass and PHA content

5 Cells were harvested by centrifugation at 8000 g for 15 min, washed with distilled water 6 and dried at 50°C to a constant weight. PHA from bacterial cells was isolated and purified by solvent extraction method⁹. Qualitative and quantitative estimation of PHA 7 was carried out by GC analysis using lyophilized cells¹⁰. The lyophilized cells (10 mg) 8 9 were subjected to methanolysis at 100°C for 140 min in 1 ml chloroform, 0.85 ml 10 methanol and 0.15 ml conc. H₂SO₄. The samples were homogenized with deionized 11 water and the bottom organic phase was used for GC analysis. Analysis was carried out 12 using a 30 m DB-1(fused silica gel-polymethyl siloxane) capillary column (internal 13 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, 14 15 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-16 17 co-HV) containing 5 mol% of hydroxyvalerate (Sigma Aldrich, USA) were used as 18 standards. Benzoic acid was used as internal standard.

19

20 FTIR and ¹H 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 4004000 cm⁻¹ in FTIR Nicolet Magna 5700 spectrophotometer (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/solubilised in chloroform and FTIR spectra were recorded as mentioned above. For
NMR analysis, 5 mg PHA samples were dissolved in CDCl₃ (1 ml) and analyzed at 400
MHz in AMX 400 (Bruker) NMR spectrophotometer (Germany).

7

8 Properties of the PHA films

9 PHA blends were prepared by homogenizing 370 mg each of PHA with PEG, glycerol or 10 PVAc in 40 ml of chloroform and the solutions were poured onto 10 x 5 cm, leveled glass 11 plate and dried at 30°C to obtain the films. PHA filmstrips (10 x 1 cm) of known 12 thickness were used for mechanical tests. Tensile strength and % elongation were 13 determined using a Universal texture measuring system (LLOYD instruments LR5K, 14 UK) at 30°C using 50 N load cell with a speed of 50 mm/ min. PHA films and the blends 15 were gold sputter coated and analyzed in a scanning electron microscope (LEO-435,

- 16 Cambridge, UK).
- 17
- 18
- 19 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 formula¹¹: Intrinsic viscosity =K x (Mw)^{0.82}, where, K-constant is 7.7x10⁻⁵. PHB, P(HB-co-HV) of 5 mol% were used as standards. 1

2 **Results and discussion**

3 Production of PHA copolymer

4 PHB, which is generally found as energy reserve of bacterial cells is brittle and co-5 polymerization 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 6 7 sucrose as the main carbon source. The polymer contained P(HB-co-HV) of 97:3 mol% 8 (Table 1). HV concentration increased to 5% by co feeding of rice bran oil during 9 cultivation. Recombinant E. coli produced 21% of scl-co-mcl PHA (71:29) copolymer 10 with glucose and decanoic acid as carbon sources. Polymer was composed of butyrate 11 hexanoate and octanoate in 71:8:21 mol%.

12

13 Detection of PHA copolymer

14 FTIR is one of the rapid and powerful tools to obtain information on polymer structure, 15 because every chemical compound in the sample makes its own distinct contribution to 16 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 17 the cells can be rapidly detected by this technique⁷. In the present study, it was observed 18 19 that FTIR spectra of PHAs containing short chain length monomers such as HB and HV) 20 and medium chain length hydroxyalkanoate monomer (hydroxyhexanoate-HHx; 21 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 22 23 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. 1 Transmittance region from 2800-3100 cm⁻¹ corresponds to stretching vibration of C-H 2 3 bonds of methyl (CH₃), and methylene (CH₂) groups. Other characteristic bands present 4 for scl PHA were 2977, 2934, 1282 (CH₃ bend), 1100, 1058 (C-O), 979 and 515. Scl-comcl PHA had the strongest methylene –C-H- vibrations near 2933 cm⁻¹. PHB standard 5 6 and PHA from R. meliloti also showed bands similar to that of scl-PHA. The results 7 indicated that by using KBr pelletized PHA, it was possible to distinguish scl from mcl 8 PHA, but within scl-PHA, HV could not be conclusively distinguished from HB.

9 Minor band differences in the absorption spectrum of homopolymer of PHB and co 10 polymers of P(HB-co-HV) in chloroform as a medium were noticed. PHB homopolymer 11 had characteristic absorption bands of 1724 and 1280, while copolymer of P(HB-co-HV) 12 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) 13 14 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 15 16 variations may be due to differences in the physical state of the PHA in chloroform. FTIR 17 spectroscopy has also been used to investigate and even predict the degree of crystallinity of PHB samples¹³. Production of P(HB-co-HV) and scl-co-mcl PHA by R. meliloti and 18 recombinant E. coli, respectively, were confirmed by ¹H NMR spectroscopy. ¹H NMR 19 20 spectrum of PHA showed signals characteristic of PHB: a doublet at 1.29 ppm, which is 21 attributed to the methyl group, a doublet of quadruplet at 2.5 ppm, which is attributed to 22 methylene group and a multiplet at 5.28 ppm characteristic of methyne group. A triplet at 23 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 literature¹⁴⁻¹⁵.

4

5 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}.

12 In the present study, PHA obtained from R. meliloti, which contained 3 mol% of HV 13 and PHA from recombinant E. coli, which contained scl-co-mcl PHA (71:29 mol%) were 14 used for blending. Standard PHB was also used for comparison. Analysis of blends by 15 FTIR using KBr pelletization indicated that the PHB was less reactive with glycerol, PEG 16 and polyvinyl acetate (Table 2). PHA from *R meliloti* and recombinant *E. coli* were more 17 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 18 19 C-O stretching. Major band shift was noticed in C=O regions for the blends. Suppression 20 of crystallinity is assigned to the formation of intermolecular hydrogen bonds between PHA carbonyls and the blend substrate used¹⁷. This is also evidenced by the FTIR band 21 22 intensity of the carbonyl stretching absorption from PHA and its blends using PVAc as a plasticizer (Table 2 and 3). The lowest band (1724 cm^{-1}) is assigned due to crystalline 23

phase and stronger absorption bands at 1735, 1743 cm⁻¹ are attributed to amorphous 1 2 phase¹⁷. This banding pattern was clear in chloroform system than in KBr pellet method. 3 Pure PHB or its blends could not be made into films. PHA blend with PVAc (1:1) 4 improved the elongation to break % (180%) compared to control film (14%, Table 4). 5 However, there was a decrease in the tensile from 28 Mpa (control) to 7 Mpa of blend 6 film. A balance may be obtained by blending known quantities of PHA and PVAc to 7 attain required tensile strength and % elongation of the film. 8 Molecular weight of PHA obtained from R. meliloti, R. meliloti + PVAc blend; recombinant E. coli, recombinant E. coli + PVAc and PVAc alone was 6.7 x 10^4 , 1.1 x 9 10^5 , 8.6 x 10^4 , 9.4 x 10^4 and 4.4 x 10^4 , respectively, which indicated modification of the 10 11 blended material that would lead to altered mechanical properties.

12

13 Scanning electron microscopy of the PHA films

14 A study on the morphology of the blends was carried out using scanning electron 15 microscopy (SEM) and it was observed that PHB exhibited a surface with high porosity. 16 Non-reactivity of PHB with PEG/glycerol/PVAc was observed as granulated/porous 17 surfaces (Fig 3). Blend of *R. meliloti* PHA with PVAc showed a homogenous background 18 layer overlaid with honeycomb like structure and each cell was thick layered compared to 19 PHB blend. This indicated enhanced reactivity of P(HB-co-HV) compared to PHB. PEG 20 blending appeared as coated surface and glycerol showed minimal blending compared to 21 control (Fig. 3D and 3F). Blend of recombinant E. coli PHA with PEG and PVAc also 22 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.

5

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1 Legends to figures:

2 Fig. 1 FTIR spectrum of KBr pelletized PHA: (A) Standard scl-PHA (PHB); (B) scl-co-



3 mcl PHA of recombinant *E. coli*.



5

- 1 Fig. 2 ¹H NMR spectrum of A) Standard P(HB-co-HV); B) PHA from *R. meliloti* C)
- 2 PHA from recombinant *E. coli;* indicating HB=Hydroxybutyrate; HV=Hydroxyvalerate;
- 3 HHx=Hydroxyhexanoate and HOc=Hydroxyoctanoate.



- 1 Fig. 3 Scanning electron micrographs showing morphology of control film and blends
- 2 (Magnification 1.00 K X): A = Control PHB; B = PHA from R. meliloti; C = PHB +
- 3 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.





1 Table-1

Bacteria	Carbon source Biomass PHA (% of Me				Mol%* of PHA components			
		(mg%)	biomass)	HB : F	IV : HH	HHx : HOc		
R. meliloti	Sucrose	300 <u>+</u> 28**	62 <u>+</u> 5	97	3	0	0	
R. meliloti	Sucrose+rice bran oil	315 <u>+</u> 7	60 <u>+</u> 5	95	5	0	0	
Recombinant E. coli	Glucose + Decanoic acid	120 <u>+</u> 14	21 <u>+</u> 3	71	-	8	21	
 *By GC analysis; **Average values of three experiments with SD; HB=Hydroxybutyrate; HV=Hydroxyvalerate; HHx=Hydroxyhexanoate; HOc=Hydroxyoctanoate 								

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

- 8 Table 2 Major FTIR wave number (cm⁻¹) band shifts of PHA blends measured after
 - Glycerol blend* PEG blend* PVAc blend* Control Nil Nil Nil Nil -Nil Nil Nil
- 9 KBr pelletization

10 1=Standard PHB; 2=PHA from *R. meliloti*; 3=PHA from recombinant *E. coli.* *PHA was

11 blended with respective plasticizers in 1:1 ratio. Bold numbers indicate strong absorption

12 bands compared to respective controls.

1 **Table 3** Major FTIR wave number (cm⁻¹) band shifts of PHA blends measured using

2 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

3 1=Standard PHB; 2=PHA from *R. meliloti*; 3=PHA from recombinant *E. coli.* *PHA was

4 blended with respective plasticizers in 1:1 ratio. Bold numbers indicate strong absorption

5 bands compared to respective controls.

6

7 Table 4 Mechanical properties of P(HB-co-HV) of R. meliloti blended with glycerol,

8 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			