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Soma Pal Saha · A. Patra · A.K. Paul

Incorporation of polyethylene glycol in polyhydroxyalkanoic acids accumulated by *Azotobacter chroococcum* MAL-201

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Abstract *Azotobacter chroococcum* MAL-201 (MTCC 3853), a free-living nitrogen-fixing bacterium accumulates poly(3-hydroxybutyric acid) [PHB, 69% of cell dry weight (CDW)] when grown on glucose and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [PHBV with 19.2 mol% 3HV] when grown on glucose and valerate. Use of ethylene glycol (EG) and/or polyethylene glycols (PEGs) of low molecular weight as sole carbon source were detrimental to *A. chroococcum* growth and polymer yields. PEG-200, however, in the presence of glucose was incorporated into the polyhydroxyalkanoate (PHA) polymer. Addition of PEG-200 (150 mM) to culture medium during mid-log phase growth favored increased incorporation of EG units (12.48 mol%) into the PHB polymer. In two-step culture experiments, where valerate and PEG simultaneously were used in fresh medium, EG was incorporated most effectively in the absence of glucose, leading to the formation of a copolymer containing 18.05 mol% 3HV and 14.78 mol% EG. The physico-mechanical properties of PEG-containing copolymer (PHBV–PEG) were compared with those of the PHB homopolymer and the PHBV copolymer. The PHBV–PEG copolymer appeared to have less crystallinity and greater flexibility than the short-chain-length (SCL) PHA polymers.

Keywords Polyhydroxyalkanoic acid · Poly(3-hydroxybutyric acid) · Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) · Polyethylene glycol · PHBV–PEG copolymers

S. P. Saha · A.K. Paul (✉)
Department of Botany, University of Calcutta,
35 Ballygunge Circular Road, 700019 Kolkata, India
E-mail: akpaul@cal3.vsnl.net.in
Tel.: +91-33-24753681
Fax: +91-33-24764419

A. Patra
Department of Chemistry, University of Calcutta,
92 Acharya Prafulla Chandra Road, 700009 Kolkata, India

Introduction

Polyhydroxyalkanoic acids (PHAs), a family of optically active polyesters, are accumulated intracellularly by microorganisms during unbalanced growth condition as a means of storing of carbon as energy reserves [1]. Over 150 different hydroxyalkanoic acid monomers have been identified as building blocks for PHAs [27]. Poly(3-hydroxybutyric acid) [PHB] was the first identified member of the PHAs family [18] and is produced by a number of bacteria. The solution cast films of PHB have brittle properties which limits the industrial uses of the polymer [13]. Introduction of 3-hydroxyvalerate (3HV) monomer into the chain of 3-hydroxybutyrate (3HB) leads to the production of copolymer PHBV having improved physico-mechanical properties and higher biodegradability [17, 21, 24]. The impact strength, flexibility, and melting temperature of PHBV vary considerably with the 3HV mole fraction present in the copolymer [15, 23].

Polyethylene glycol (PEG), a neutral water-soluble polyether is relatively non-toxic to cellular systems and is absorbed into proteins and the phospholipid head group [8, 29]. PEG is used in processes such as protein modification, cell fusion, organ preservation, etc. The antimicrobial properties of PEG-400 against pathogenic bacteria also were reported [11]. The interaction of PEG with PHA synthesis has been investigated to induce variation in polymer composition and molar mass of short-chain-length (SCL) PHA. In earlier studies, PEG was effectively utilized to control molar mass of SCL PHA produced by *Ralstonia eutrophus* [25], *Alcaligenes latus* [2, 3], and short-chain and medium-chain-length (MCL) PHA from *Pseudomonus oleovorans* [4]. Addition of PEG into culture medium causes a change in PHA biosynthesis involving the enzyme system and results in the incorporation of ethylene glycol (EG) monomer repeat units into the PHA polymer. Formation of PHA–PEG diblock where carboxylate (–COOH) terminus of PHA chains are covalently linked by an ester bond to a PEG chain was established [26]. Furthermore, PEG in

the media results in osmotic stress that is tolerated either by the accumulation of a solute such as potassium and calcium ions or by a change in cell morphology.

Azotobacter is a bacterium that can accumulate both PHB and PHBV [19, 20]. During the present study, attempts were made to produce PEG-containing PHAs (PHBV–PEG inclusions) by *Azotobacter chroococcum* MAL-201 (MTCC 3853) utilizing glucose, valerate, EG, and PEG following both single- and multi-step cultivation procedures. The physico-mechanical and thermal properties of the polymers were determined and compared with the PHB homopolymer and the PHBV copolymer produced by the strain MAL-201.

Materials and methods

Bacterial strains and medium

A. chroococcum MAL-201 (MTCC 3853), a PHA producing non-symbiotic nitrogen-fixing bacterium, was used through out the study. Norris nitrogen-free medium as modified by Stockdale et al. [28] containing 2% (w/v) glucose was principally used for growth and polymer production and the culture was maintained on slopes of the same medium.

Fermentation and stepwise cultivation

In a single-step cultivation process, the nitrogen-free medium containing 2% (w/v) glucose (100 per 250 ml Erlenmeyer flask) was inoculated with freshly prepared inoculum at 4% (v/v) level and incubated at 32°C on a rotary shaker (120 rpm) for 27 h [19]. The inoculum was prepared by growing the organism in the same medium for 24 h. Sodium valerate, EG, and PEG of different molecular weights were added to the medium as cosubstrate as and when required.

In a two-step cultivation process, cells from 12-h-old culture grown in glucose (2%, w/v) containing medium were harvested aseptically by centrifugation, washed with sterile carbon-free medium and transferred to sodium valerate (0.2%, w/v) containing medium and PEG was added after 6 h of incubation. Cell mass was harvested after a total of 27 h incubation.

In a three-step cultivation, the first step (12 h) was to initiate growth, the second step (next 6 h) was directed towards the synthesis of PHBV using 0.2% sodium valerate. In the third and final step, the biomass from the second step was harvested and transferred aseptically to PEG-containing carbon-free medium and incubated for an additional 9 h to facilitate incorporation of EG units into the copolymer.

Estimation of growth

Growth of the organism was determined either by measuring optical density of the medium at 540 nm or by measuring the dry weight of biomass after a thorough washing with deionized water for several times.

Assay of the polymer

For quantification and compositional analysis, washed and oven-dried cells were subjected to methanolysis in acidified methanol (15% H_2SO_4, v/v): chloroform mixture (1:1) at 100°C as described by Brandl et al. [9]. The methyl ester derivatives in the chloroform fraction were analyzed using Chemito 8510 gas chromatograph equipped with a SE-30 stainless steel column (13′1/8″) and a flame ionization detector. Nitrogen (2.5 ml/min) was used as a carrier gas. For calibration, Na-DL-3-hydroxybutyric acid, PHB, PHBV standards from Sigma, USA were used.

Isolation of polymer

The intracellular polymer was extracted from thoroughly washed and oven-dried biomass with chloroform at 60°C for 2 h following the method of Ramsay et al. [22]. The polymers were isolated by precipitation with diethyl ether and their physical properties were determined.

Characterization of polymers

The ^{1}H-NMR spectra of purified PHBV–PEG copolymers and its acetylated derivative in $CDCl_3$ were recorded at 300.13 MHz on a Bruker AV300 Supercon NMR spectrometer. Acetylation of the polymer was performed by treating its CH_2Cl_2 solution with acetic anhydride and pyridine. The molecular weight of PHBV–PEG copolymers was determined through intrinsic viscosity [η] (dl/g) measurement using Mark–Howink equation: $[\eta] = 7.7 \times 10^{-5} \times M^{0.82}$ [10].

The viscometric measurements were performed in chloroform solution at 30°C by Ubbelohde's dilution viscosimeter [7]. Elemental analyses of samples were carried out on a Perkin Elmer Elemental Analyzer, model 2400 series II. Thermal characterization was done with a thermo gravimetric analyzer (TGA) Metler Toledo TGA/sDTA 851^e and a differential scanning colorimeter (DSC), Metler Toledo 827 thermal analyzer with a scanning temperature ranged from −20 to +220°C and at a scan rate of 10°C/min. The X-ray diffraction (XRD) pattern of polymer sheets (24 h old) was recorded in a SEIFERT XRD diffractometer model 3000P (Germany). Nickel filtered CuK_α radiation ($\lambda = 0.154$ nm; 35 kV; 30 mA) was used at room temperature. The tensile strength of polymer sheet (0.75 mm thick and 4 mm width) was tested using Instron model 4301 (Germany) with a crosshead speed 20 mm/min.

Results

Effect of EG and PEG on growth and polymer production

The growth and polymer production by MAL-201 in nitrogen-free Stockdale medium supplemented with EG (50 mM) and PEG-200 (50 mM) were less compared to controls (Fig. 1). Incorporation of PEG in the accumulated polymer was better (2.5 mol%) than the incorporation of EG which appeared to be negligible. Moreover, it was noticed that EG and PEG when used as sole carbon source (instead of glucose) failed to support growth and polymer accumulation by the strain.

The time course of growth of MAL-201 as determined by optical density changes revealed a minor variation in the growth patterns in glucose containing medium as well as in the media supplemented with PEG-200, PEG-400, and PEG-1000, each at the final concentration of 50 mM (Fig. 2). Regardless of yield, polymer accumulation by the isolate in each case was more or less parallel to growth (Fig. 3). However, the polymer yield increased with an increase in the molecular weight of PEG although they were much lower than that of the control.

Effect of PEG on copolymer production

Addition of sodium valerate (0.2%, w/v) at mid-log phase growth of the organism in PEG-200 (50 mM) supplemented medium indicated that the PEG of low molecular weight favored the EG monomer incorporation (2.8 mol%) with the 3HV content (19.63 mol%) in the polymer being similar to that of control. With an increase in molecular weight of PEG, the 3HV and EG content decreased significantly although the polymer yield with PEG-1000 was much higher than those obtained with PEG of low molecular weight (Table 1). Incorporation of PEG into the PHA polymer was determined by ^{1}H-NMR spectroscopy of acetylated product of PHBV–PEG copolymer. The signals of methylene protons of PHB, PHBV, and PHBV–PEG in $CDCl_3$ solution at $\delta = 1.25$, 0.9, and 3.6–3.8, respectively, were determined by ^{1}H-NMR data analyses (Fig. 4). Two small resonances at $\delta = 2.1$ (s) and 4.25 (m), respectively, represent the methyl of acetyl now attached to one end of PEG and methylene groups (of covalent bonds) bearing acetate and between PHBV and PEG chains.

Standardization of PEG addition

Addition of PEG-200 (50 mM) to glucose containing nitrogen-free medium at different phases of growth promoted growth, polymer accumulation, and yield. Maximum polymer production (67% of CDW) was obtained when PEG-200 was added after 24 h incubation. The EG content in polymer was 4.53 mol% when PEG was added to the culture at late log phase (18 h) of growth (Table 2).

Effect of PEG concentration

A significant increase in PEG-200 uptake in terms of mol% EG in PHA polymer was noted with an increase in concentration (50–150 mM) of PEG-200 (Table 3). The maximum incorporation of EG monomers (12.85 mol%) along with 14.22 mol% of 3HV in PHA was obtained with PEG-200 at 150 mM concentration. Further increases in PEG concentration inhibited growth, reduced PHA production, and 3HV incorporation (Table 3).

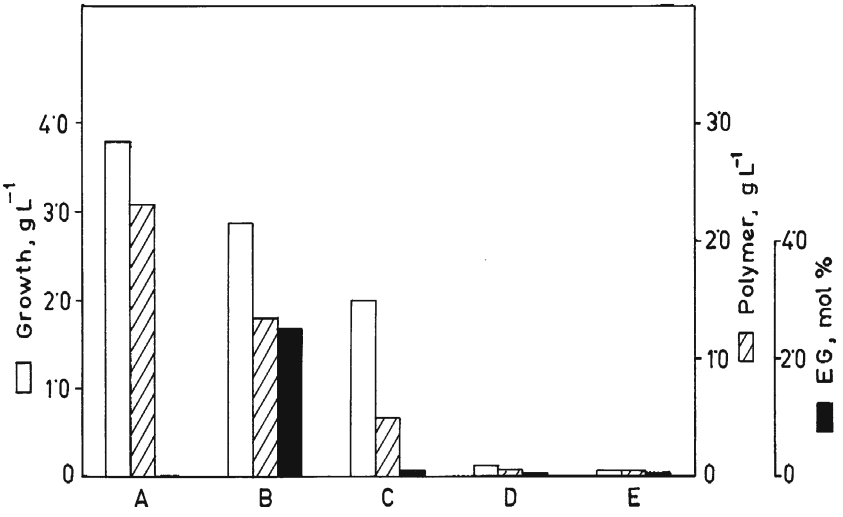

Fig. 1 Effect of ethylene glycol (50 mM) and PEG-200 (50 mM) on growth (*open square*), polymer yield (*hatched square*), and EG content in polymer (*filled square*) produced by *A. chroococcum* MAL-201. Carbon substrates (w/w) used in modified Norris N_2-free medium are: *A* (glucose, 2.0%); *B* (glucose + PEG-200, 50 mM); *C* (glucose + EG, 50 mM); *D* (PEG-200, 50 mM); and *E* (EG, 50 mM)

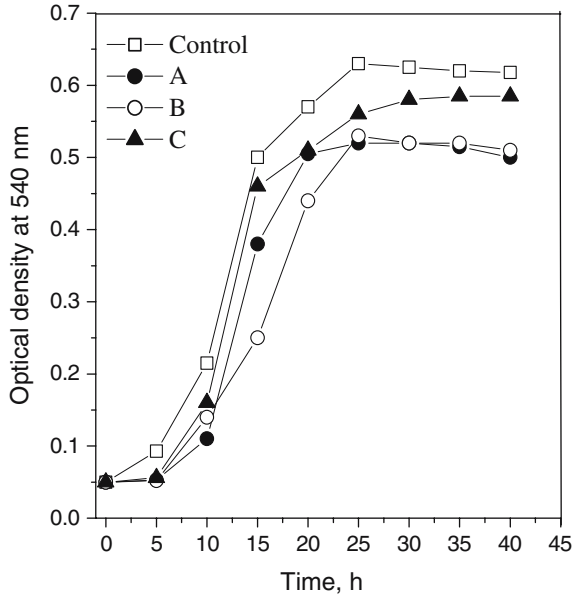

Fig. 2 Effect of PEG of different molecular weights on growth of *A. chroococcum* MAL-201 in modified Norris N_2-free medium at 32°C under shake flask condition. Control (medium without PEG); *A* (with PEG-200); *B* (with PEG-400), and *C* (with PEG-1000). PEG (50 mM each) was added at 0 h

Production of copolymers by stepwise cultivation

In stepwise cultivation experiments, the organism was grown (12 h) initially in nitrogen-free medium with glucose as the sole carbon source. In single-step cultivation, addition of valerate (0.2%, w/v) and PEG (150 mM) to the 12 and 18-h-old growing culture resulted in a polymer content of 56.0% of CDW with 3HB (76.8 mol%) and 3HV and EG monomers being 11.3 and 11.9 mol%, respectively (Table 4). In two-step cultivation, harvested and washed cells from initial growth phase were transferred to fresh medium containing valerate and PEG being added to it after 6 h of growth. This resulted in an increase in the polymer production and accumulated polymer contained maximum 3HV, 18.05 mol% and EG, 14.78 mol%. The three-step cultivation process (where glucose and then valerate grown cells were finally transferred to the fresh medium containing PEG as a sole carbon source), however, failed to show any variation in polymer production and composition.

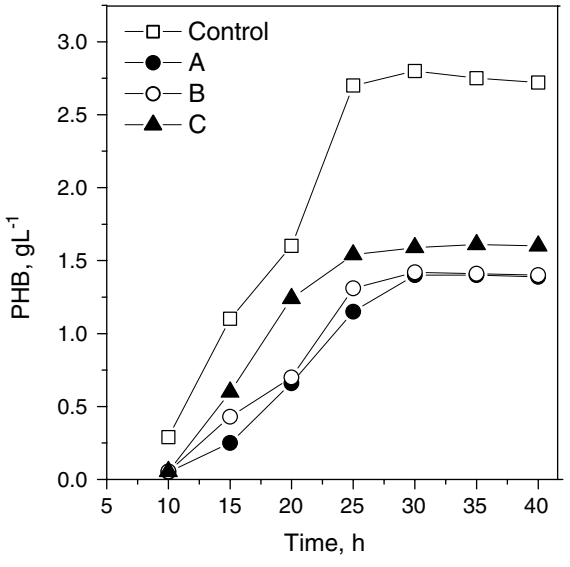

Fig. 3 Effect of PEG of different molecular weights on PHB yields by *A. chroococcum* MAL-201. Cultural conditions were same as shown in Fig. 2

Table 1 Effect of PEG of different molecular weights on growth and polymer yield by *A. chroococcum* MAL-201

PEG[a] (molecular weight)	Growth[b] (g/l)	Polymer yield[c] (g/l)	Monomer[d] (mol%)		
			3HB	3HV	EG
Control (without PEG)	3.8	2.66	79.26	20.70	ND
PEG-200	2.9	1.40	77.57	19.63	2.80
PEG-400	3.1	1.50	78.00	15.80	2.20
PEG-1000	3.6	1.80	84.64	9.60	1.76

[a]PEG of different molecular weights, each at 50 mM concentration was added individually to the culture medium at 0 h except in control set
[b]Cells were harvested after 27 h of incubation at 32°C under shake flask condition (120 rpm). Yield of biomass was calculated from dry weight measurement
[c]Yield of the polymer was estimated by calculating the polymer content per gram of cell and biomass yield per liter. Total polymer content of the cell mass was calculated by using gas-chromatograph following the method of Brandl et al. [9]
[d]The mol% of monomers present in polymer were calculated following ^{1}H-NMR (at 300.13 MHz) data analyses of the purified sample

Polymer characterization

Three different polymer samples namely, PHB homopolymer, PHBV copolymer (19 mol% 3HV), and PHBV–PEG copolymer (18 mol% 3HV and 14 mol% EG as represented in Table 4) were isolated, purified, and characterized in detail. The results are presented in Table 5. Elemental analyses indicated little variation in C, H, and O content of the polymers. The PHB having a molecular weight, 2,937 kDa showed more thermal stability (>98% weight loss in a single step at 280°C) and higher melting temperature than those of the PHBV and PHBV–PEG copolymers. Incorporation of EG units into the polymer reduced the molecular weight, density, tensile strength, and hardness, but water uptake efficiency of the PHBV–PEG copolymer was increased by 12 and 25% compared to the PHBV copolymer and PHB homopolymer, respectively.

Discussion

A. chroococcum MAL-201 has been reported to produce PHB in up to 69% of CDW during its exponential

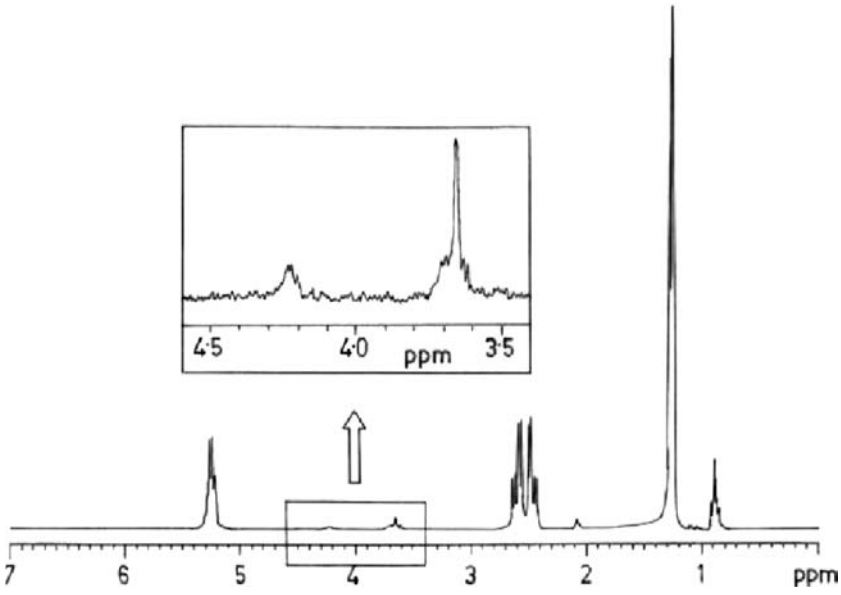

Fig. 4 300.13 MHz ^{1}H-NMR spectrum of acetylated derivative of PHBV–PEG copolymer isolated from *A. chroococcum* MAL-201 cells, grown in single-step culture in modified Norris N_2−free medium containing glucose (2%, w/v), sodium valerate (0.2%, w/v), and PEG-200 (50 mM)

growth phase in nitrogen-free glucose containing medium [20]. It can also produce a copolymer of PHBV with sodium valerate as cosubstrate (unpublished data). The present findings clearly indicated the inability of this organism to utilize EG and PEG as sole carbon sources although these substrates were tolerated in the presence of glucose (Fig. 1). The greater tolerance of MAL-201 to higher molecular weight PEG (Fig. 2) is comparable to *R. eutrophus*, which tolerated 10% (w/v) PEG-10000 with only minor variation in cell yields [26]. Growth and polymer yields were most inhibited by PEG-200 (Fig. 3). Though PEG-200 (50 mM) caused nearly a 45% reduction in polymer yield but its incorporation in polymer was maximal (2.8 mol%) and the chance of EG incorporation increased with the increase of PEG concentration in medium (Table 3). Addition of PEG at the start of culture growth had a detrimental effect on polymer yield. This could be minimized by the addition of PEG at the mid-log phase of growth, which is also associated with polymer synthesis. It was evident that EG incorporation occurred mostly during the synthesis of PHA polymers or when a good amount of PHA polymer present in cell, suggesting possible interaction of PEG with PHA synthase enzyme and the polymer itself. The miscible nature of PEG with PHA was reported earlier with *R. eutropha* [5].

Effective polymer production and incorporation of PEG could be achieved by stepwise culture methods. It was apparent that *A. chroococcum* MAL-201 when grown in glucose containing medium produced only

Table 2 Optimization of time for the addition of PEG-200 to the culture medium of *A. chroococcum* MAL-201

Time[a] (h)	Growth[b] (g/l)	Polymer[c] (g/l)	Polymer[c] (%, CDW)	EG[d] (mol%)
0	2.47	0.97	48.90	2.40
6	2.49	1.00	49.40	2.45
12	2.52	1.18	51.20	4.20
18	2.55	1.29	58.60	4.53
24	2.58	1.78	67.00	Trace

[a]PEG-200 at a concentration of 50 mM was added to the culture medium at different hours of growth of the organism
[b]Cells were harvested after 27 h of incubation at 32°C under shake flask condition (120 rpm). Yield of biomass was calculatedfrom dry weight measurement
[c]Yield of the polymer was estimated by calculating the polymer content per gram of cell and biomass yield per liter. Totalpolymer content of the cell mass was calculated by using gas-chromatography following the method of Brandl et al. [9]
[d]The mol% of monomers present in polymer were calculated following 1H-NMR (at 300.13 MHz) data analyses of the purifiedsample

Table 3 Effect of PEG-200 concentration on growth and accumulation of polymer by *A. chroococcum* MAL-201

PEG (mM)	Growth[a] (g/l)	Polymer[b] (%, CDW)	Mol%[c]			Percentage inhibition of growth[d]
			3HB	3HV	EG	
0	2.6	69.29	80.78	19.22	ND	ND
50	2.5	58.35	82.93	12.51	4.55	3.85
100	2.2	56.60	76.74	13.44	9.81	15.0
150	2.05	47.59	70.93	14.22	12.85	21.15
200	1.98	46.00	77.59	10.98	11.42	23.84
250	1.75	40.40	75.84	11.5	12.65	32.69

[a]Cells were harvested after 27 h of incubation at 32°C under shake flask condition (120 rpm). Yield of biomass was calculatedfrom dry weight measurement
[b]Total polymer content of the cell mass was calculated by using gas-chromatography following the method of Brandl et al. [9]
[c]The mol% of monomers present in polymer were calculated following 1H-NMR (at 300.13 MHz) data analyses of the purifiedsample
[d]The growth of the bacterium without PEG was considered as control. The dry weight of the biomass grown with respective concentration of PEG was deducted from the control and its percentage inhibition of growth was calculated

Table 4 Growth, polymer composition, and its yield during single- and multi-step cultivation of *A. chroococcum* MAL-201

Culture step	Growth[a] (g/l)	Polymer yield[b] (g/l)	Mol%[c]		
			3HB	3HV	EG
Single-step	2.5	1.40	76.80	11.30	11.90
Two-step	2.54	1.50	67.17	18.05	14.78
Three-step	2.53	1.51	70.49	14.01	15.50

[a]Cells were harvested after 27 h of incubation at 32°C under shake flask condition (120 rpm). Yield of biomass was calculatedfrom dry weight measurement

[b]Yield of the polymer was estimated by calculating the polymer content per gram of cell and biomass yield per liter. Totalpolymer content of the cell mass was calculated by using gas-chromatography following the method of Brandl et al. [9]

[c]The mol% of monomers present in polymer were calculated following 1H-NMR (at 300.13 MHz) data analyses of the purifiedsample

acetoacetyl–CoA for 3HB biosynthesis and the presence of valerate as cosubstrate induced the synthesis of 3-ketopentanoyl-CoA for 3HV synthesis for production of PHBV copolymer as reported for *A. vinelandii* UWD [19].

In single-step cultivations with glucose the incorporation of 3HB, 3HV, and EG were achieved. Choi et al. [12] used a two-step culture method for the production of P(3HB-co-4HB) copolymer rich in 4HB by *Hydrogenophaga pseudoflava*. Similarly, the accumulation of 3-hydroxyalkanoic acids other than 3HB was accomplished by Du et al. [14] in *R. eutropha*. Likewise, for *A. chroococcum* MAL-201 the two-step cultivation promoted growth, polymer yield as well as incorporation of EG into the growing polymer when PEG was added at the PHA synthesis phase and EG uptake increased slightly more when PEG was added to fresh medium during the three-step cultivation (Table 4).

Introduction of PEG into PHA polymers caused significant changes in polymer molecular weight along with thermo-mechanical properties (Table 5). Shi and Gross [25] attributed the reduction of PHAs molecular weight to PEG limiting the polymer chain length. Acetylation of hydroxyl group at the free end of PEG segment (resonance at $\delta = 2.1$ for methyl protons of acetate unit formed as in Fig. 4) suggests the PEG chain attachment with a covalent bond (resonance at $\delta = 4.25$) at the terminal position of a PHBV chain. The formation of low molecular weight EG containing copolymers by MAL-201 seemed to be the results of interaction of PEG with the PHA molecules itself as for *R. eutropha* also [26]. The presence of hydroxyl groups in the PHBV–PEG copolymer results in a higher percentage of oxygen and hydrogen in polymer including low intrinsic viscosity and density along with increased water uptake capacity. Thermo-gravimetric analyses showed >98% weight loss for PHB and PHBV as a single exotherm between 270 and 280°C [16], where as the decomposition of PHBV–PEG occurred at 255°C temperature with a low melting enthalpy as determined by calorimetric assay (Table 5). The 24 h-old PHB and PHBV polymer sheets showed high crystallinity, typical of PHA_{SCL} [6, 24] whereas the PHBV–PEG sheet of same age was comparatively soft having crystallinity 32% only. It was evident that with a reduction in molecular weight (1,803 kDa), brittleness and low tensile strength (17.5 MPa), the PHBV–PEG copolymer gave a better quality polymer similar to elastomeric PHA_{MCL} obtained from the cells of *P. oleovorans* grown on higher alkanoates [9]. Therefore, the introduction of PEG into *A. chroococcum* MAL-201 culture could be considered as one approach for the production of PHA_{SCL}–PEG polymer, which might be a better option for PHA_{SCL} for commercial use.

Table 5 Characterization of purified PHB homopolymer, PHBV copolymer (19 mol% 3HV), and PHBV–PEG copolymer (18 mol% 3HV and 14 mol% EG) isolated from *A. chroococcum* MAL-201

	PHB	PHBV	PHBV–PEG
Elemental analyses	C-55.48 H-36.36 O-8.16	C-58.60 H-34.15 O-7.25	C-53.34 H-39.16 O-7.50
Molecular weight[a] (kDa)	2,937	2,398	1,803
Intrinsic viscosity[a] [η] (dl/g)	15.5	13.2	10.2
Density[a] (g/cm^3)	1.656	1.692	1.252
Thermal degradation[b] (°C)	280	268	255
Weight loss during thermal degradation[b] (%)	98.47	98.9	84.11
Melting temperature[d] (°C)	178.73	168.5	145
Melting enthalpy[d] (ΔH_m) (J/g)	91.0	76.30	68.55
Crystallinity[c] (%)	68.35	64.66	32.54
Tensile strength[a] (MPa)	34.0	29.0	17.5
Hardness, shore[a] *D*	55.0	51.0	7.4
Water uptake[a] (%)	0.032	0.036	0.04

[a]Intrinsic viscosity, molecular weight, density, mechanical properties, and water uptake capacity of samples were estimated at 30°C

[b]Data of thermal degradation and percentage of weight loss were obtained from TGA analyses

[c]Percentage of crystallinity of isolated polymers were calculated from XRD analyses

[d]Data of melting temperature and melting enthalpy were obtained from DSC experiments

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