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Comparative Analysis of Different Properties of Polyhydroxyalkanoates Isolated from Two Different Bacterial Strains: *Alkaliphilus oremlandii* OhILAs and Recombinant *Escherichia coli* XL1B

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ABSTRACT: We synthesized poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3-HB-*co*-3-HV)] copolymer having different contents of 3-hydroxyvalerate (3-HV) units (16.04, 16.3, 24.95, 25.62, and 16.52 mol % 3-HV) with different yields of polyhydroxyalkanoates (PHAs) by feeding with different cooking oils and with *Alkaliphilus oremlandii* OhILAs strain. The PHA production efficiency of the *Alkaiphilus* strain was compared with that of the control strain, *Bacillus cereus*. The synthesis of each PHA biopolymer was performed with different toxic spent oils as the sole carbon source in an oil-in-water-based microemulsion medium. We observed that the productivity of the poly(3-hydroxybutyrate) [P(3-HB)] copolymer from the *Alkaliphilus* strain was higher than those of the PHAs isolated from *B. cereus* and the *Escherichia coli* XL1B strain. The synthesized PHA copolymers were characterized by ^{1}H-NMR and Fourier transform infrared (FTIR) spectroscopy. In the ^{1}H-NMR spectra, a doublet resonance peak at 1.253 ppm of the/ methyl protons of the 3-hydroxybutyrate (3-HB) side group and one at 0.894 ppm due to the methyl protons of the 3-HV side group indicated the presence of 3-HB and 3-HV units in the copolymer. The chemical shift values at 1.25 and 2.2 ppm, due to the resonance absorption peaks of the methyl protons and methylene protons, confirmed the synthesis of the P(3-HB) homopolymer. From the FTIR spectra, a strong C═O stretching frequency in the range of 1745–1727 cm^{-1}, together with strong C—O stretching bands near 1200 cm^{-1} and a strong band near 3400 cm^{-1}, confirmed the synthesis of P(3-HB-*co*-3-HV) and P(3-HB). Thus, waste cooking oil as a substrate provided an alternate route for the formation of P(3-HB-*co*-3-HV) and P(3-HB) by *Alkaliphilus* and *E. coli* strains, respectively. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 41080.

KEYWORDS: biocompatibility; biodegradable; biosynthesis of polymers

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INTRODUCTION

Poly(3-hydroxybutyrate) [P(3-HB)] {with its poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3-HB-*co*-3-HV)] copolymer} is a common and versatile polymer of polyhydroxyalkanoate (PHA); it was first described by Lemoigne,[1] a French scientist, in the year 1925. Because various bacterial strains among Gram-positive and Gram-negative bacteria have been identified to accumulate PHAs with different chain lengths both in aerobic and anaerobic conditions and in the presence of excess carbon and limited nutritional factors, such as nitrogen, phosphorus, and potassium, in the presence of excess carbon.[2] P(3-HB) can readily be produced from renewable resources, such as sugars, fatty acids, and plant oil, as intracellular storage compounds; this provides a reserve of carbon and energy for microorganisms. Studies have also shown that some bacteria can accumulate high levels of PHA per cellular dry mass for the intracellular storage of carbon and as an energy source for their survival.[3,4]

Researchers are more prone to improving the yield of biobased plastics by DNA modification[5] technology to reduce production costs and carbon dioxide emissions. Li et al.[6] and Theodorou et al.[7] have described a method for the augmentation of the yield of a polymer by the introduction of direct genetic changes by recombinant DNA technology in host microbial cells. The active intermediates of PHA synthase of the recombinant strain are also a major factor in the synthesis of practical bioplastics.[4] In addition to the low-cost and high-productivity production of P(3-HB) from recombinant bacterial strains, researchers have also been able to store biocopolymers in the cytoplasm of genetically modified bacterial strains by the addition of sufficient precursors.[8,9] P(3-HB-*co*-3-HV) is a copolymer formed by the monomers 3-hydroxyl butanoic acid and 3-hydroxyl pentanoic acid or valeric acid. The formation of the P(3-HB-*co*-3-HV) copolymer with the addition of these precursors (valeric acid, propionic acid, etc.) has been analyzed by many researchers.[10] Numerous bacteria synthesize and accumulate P(3-HB) as a carbon and energy-storage material, but the direct synthesis of the P(3-HB-*co*-3-HV) copolymer from the carbon source without any precursors has been found to be limited.[10]

The copolymer P(3-HB-co-3-HV) is commercially more interesting than P(3-HB) because of several properties, particularly, its melting temperature (T_m), crystallinity (X_c), plasticity, and biodegradability,[11,12] and especially its bioapplications.[13–17] The biosynthesis of P(3-HB-*co*-3-HV) in bacteria involves two parallel pathways. One leads to the formation of a C_4 monomer [3-hydroxybutyrate (3-HB)], and other leads to the formation of a C_5 monomer [3-hydroxyvalerate (3-HV)] in the copolymer. The C_5 monomer is formed through the condensation of acetyl-*co*-enzyme A (acetyl–CoA) and propionyl–CoA by the action of β-ketothiolase.[18,19]

The biosynthesis of P(3-HB-*co*-3-HV) through a genetically modified mutant strain is a novel but more time-consuming and high cost-effective phenomenon. Therefore, the synthesis of a highly productive P(3-HB-*co*-3-HV) copolymer with a maximum content (molar percentage) of 3-HV units from the cheap carbon source is a primary concern of researchers (Scheme 1).

Waste cooking oil is a cheap carbon source; it is toxic for healthy tissues because of its structural cis-to-trans transformation of fatty ester when it is used repeatedly for cooking.[20] Through the collection of the waste cooking oil, a group of authors[20] was able to synthesize PHAs with different microbial strains. At about 68 wt %, P(3-HB) was synthesized from various plant oils by *Cupriavidus* species USMAA2–4. *P. aeruginosa* 47T2 was used to synthesize 28.2% PHA (by dry cell weight) from waste frying oil.[21] *Pseudomonas guezennei biovar. tikehau* and *Burkhol deria* species USM(9JCM 15050) were also capable of producing PHA from waste oil. The production of PHA and its copolymer from cooking oil at a high yield is very difficult because the oil is immiscible in a hydrophilic aqueous environment. Therefore, our main objective was to synthesize a high yield of the P(3-HB-*co*-3-HV) copolymer from different waste cooking oils with the formation of a dispersible oil-in-water (O/W) microemulsion by an *Alkaliphilus oremlandii* strain and compare its production efficiency with that of control bacterial strains, *Bacillus cereuc* and an *Escherichia coli* XL1B strain. The *E. coli* XL1B strain was also selected for the comparative study because the probability of intracellular PHA polymer degradation was negligible because of the absence of a depolymerase system in their membrane. Therefore, the purity of the isolated polymer seemed to be higher than that of the *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) and *Cupriavidus necator* strains.[22,23]

EXPERIMENTAL

Materials and Methods

Materials. Chloroform, methanol, and nutrient broth were purchased from Merck, India, Ltd.

Microorganisms. Recombinant *E. coli* XL1-Blue (pSYL107) was used for the production of P(3-HB). The stable, high-copy-number plasmid pSYL107 harboring the *R. eutropha* PHA synthesis genes and the *E. coli ftsZ* gene were used for the intracellular production of P(3-HB) and were described previously by Choi and Lee.[24]

PHA Production Strain

Isolation of the *A. oremlandii* Strain from Rhizosphere Soil and the Preparation of the Biomass for P(3-HB-*co*-3-HV) Production. Rhizosphere soil was collected from the University of Calcutta campus (Kolkata, India), and the *A. oremlandii* OhILAs strain was isolated with a serial dilution method. The specific media used for the isolation of the *A. oremlandii* OhILAs strain was composed of K_2HPO_4 (1.0 g), $MgSO_4{\cdot}7H_2O$ (0.2 g), $FeSO_4{\cdot}7H_2O$ (0.05 g), $CaCl_2{\cdot}2H_2O$ (0.1 g), $Na_2MoO_4{\cdot}2H_2O$ (1 mg), and glucose (10 g) on a per liter basis. The pH of the media was maintained at 7.0, and the isolation was done at 30°C.

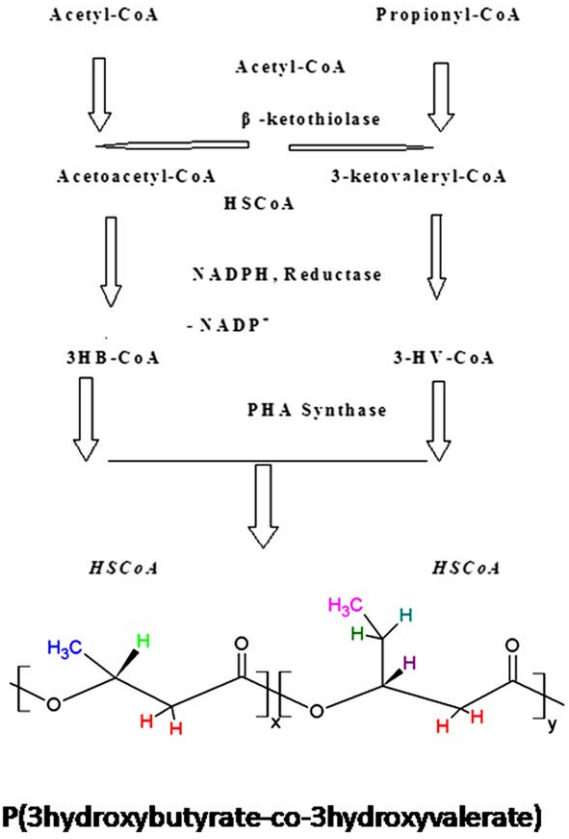
The microorganisms were subcultured at a specified duration and stored at 4°C. The isolated colonies of the *A. oremlandii* OhILAs strain were tested for PHA production with the previous broth medium in the presence of a limited nitrogen source, and the one with best production yield was selected for the rest of the experimental studies.

Inoculation of the *Alkaliphilus* Strain and Recombinant *E. coli* XL1B Strain into a Common Medium and Biopolymer Production. The *A. oremlandii* and *E. coli* strains were inoculated separately in a common growth medium. The medium consisted of 0.3 g of NH_4NO_3, 0.064 g of K_2HPO_4, 0.02 g of KH_2PO_4, 0.04 g of $MgSO_4{\cdot}7H_2O$, 0.01 g of $CaCl_2{\cdot}2H_2O$, 1 mg of $FeS0_4{\cdot}7H_2O$, 0.6 mg of $Na_2MoO_4{\cdot}2H_2O$, and 0.05 g of sodium citrate in 100 mL of distilled water. Waste cooked rapeseed oil [20 g/L] was used as the carbon source. With different sets of composition, various growth media were prepared, and all media were adjusted to pH values of 5, 6, 7, 8, 9, and 10 with dilute NaOH and HCl. The O/W microemulsion was prepared with a surfactant and cosurfactant to decrease the interfacial tension and also to increase the droplet size.[25] Then, it was sterilized in an autoclave at a pressure of 50 psi and at a temperature of 121°C for 65 min. Cells were grown in an orbital shaker (105 rpm) at a temperature range of 25–43°C and harvested after 96 h of incubation by centrifugation at 6000 g for 10 min. By applying the optimum conditions, we also inoculated the bacterial strains with different spent oils (soya oil, palm oil, coconut oil, mustard oil, and rapeseed oil) to obtained different PHA biopolymers with various yields. Finally, the cell pellet was resuspended and washed twice with saline water before cell disruption in a sodium hypochlorite solution.

Incubation of the Control Bacterial Strain, *Bacillus cereus*, for the Production of P(3-HB-*co*-3-HV). The PHA production by the bacterial strain *B. cereus* MTCC 4079 was done in an O/W microemulsed growth medium containing the following nutrients: 2.5 g/L yeast extract, 4.0 g/L tryptone, 1.25 g/L NaCl, and 2% cooked vegetable oil as the sole carbon source. The optimum pH value of the growth medium was adjusted to 7 with diluted NaOH or diluted HCl. The sterilized medium was inoculated with 2% v/v of 24 h old inoculum and incubated at 30°C in a shaker at 150 rpm for 72 h. The bacterial biomass and PHA yield were determined during the optimization of each parameter according to the method described by Masood et al.[26]

Growth Media and Culturing for the *E. coli* Strain

The culturing of the *E. coli* XL1B strain was performed in 100 mL of solution of Luria Broth, which consisted of yeast extract (1 g/L), $(NH4)_2SO_4$ (0.2 g/L), $MgSO_4$ (0.1 g/L), $Na_2HPO_4{\cdot}12H_2O$ (0.45 g/L), KH_2PO_4 (0.15 g/L), Fe (III)—NH_4 citrate (0.005 g/L), $CaCl_2$ (0.002 g/L), and 1 mL/L of a trace element solution consisting of $ZnSO_4{\cdot}7H_2O$ (10 mg/L), $MnCl_2{\cdot}4H_2O$ (3 mg/L), H_3BO_3 (30 mg/L), $CoCl_2{\cdot}6H_2O$



Scheme 1. The biosynthetic pathway of P(3HB-*co*-3HV) in microorganisms using renewable carbon source. HSCoA: Co-enzyme, NADP: Nicotinamide adenine dinucleotide phosphate, NADPH: reduced Nicotinamide adenine dinucleotide phosphate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(20 mg/L), $CuSO_4 \cdot 5H_2O$ (1 mg/L), $NiCl_2 \cdot 6H_2O$ (2 mg/L), and $NaMoO_4 \cdot 2H_2O$ (3 mg/L). The media was then sterilized in an autoclave at a pressure of 50 psi at a temperature of 121°C for 65 min, and the culture medium was grown at 37°C for 24 h in an orbital shaker (105 rpm).

Preparation of the O/W Microemulsion

Microemulsions are homogeneous, transparent liquid dispersions of water and oil that make up a thermodynamically stable system having a droplet diameter in the range 10–100 nm. The stability of the microemulsion system is dependent on the concentration of each component (the nature and amount of the oil, surfactant/emulsifying agent, and cosurfactant) present in the system. The most fundamental principle of the preparation of a microemulsion is the reduction of the high interfacial tension between the O/W phase. Because of this fact, an emulsifying agent is used to sufficiently reduce the O/W interfacial tension, that is, lower the energy required to increase the surface area so that the spontaneous dispersion of water or oil droplets occurs and the system becomes thermodynamically stable. The cosurfactant is also amphiphilic, with an affinity for both the oil and aqueous phases, and partitions to an appreciable extent into the surfactant interfacial monolayer present at the O/W interface. The simultaneous formation of the microemulsion is accompanied by an increase in the interface area (ΔA) and the formation of free energy (ΔG). The change in the interfacial tension with the formulation of the O/W microemulsion can be represented as follows:

$$\Delta G = \gamma \Delta A - T\Delta S$$

where γ is the surface tension of the O/W microemulsion, ΔS is the change in entropy, and T is the temperature on the absolute scale.

Selection of the Oil Phase

Different spent oils, including rapeseed oil, palm oil, mustard oil, soya oil, and coconut oil, were used to formulate the O/W microemulsions.

Surfactant and Cosurfactant

The criteria for the selection of the surfactant were its efficient emulsification capacity and nontoxic nature. Guar gum, a natural carbohydrate, was taken for this purpose, in which it worked as a good emulsifying agent and a nutrient of bacterial growth. 2-Propanol and poly(ethylene glycol) 400 were used as cosurfactants.

Preparation of the Microemulsion

The O/W microemulsion was prepared with a standard method proposed by Schulman et al.[27] The mixture of the surfactant and cosurfactant and the spent oil were titrated with distilled water to prepare the O/W microemulsion. Guar gum was used as an emulsifying agent, and 2-propanol was used as a cosurfactant. First, 0.15–0.2% guar gum, 2 g of cooking oil, and 7–8 mL of 2-propanol were mixed. Then, this mixture was titrated by distilled water with continuous stirring; this resulted in the formulation of a transparent and homogeneous microemulsion. The preparation of the O/W microemulsion with a triple-blade homogenizer is shown schematically in Figure 1, and the composition was then centrifuged to remove the insoluble large particles of guar gum at 6000 rpm.

Optimization of the pH, Temperature, and Incubation Periods

The optimization of different parameters, such as the pH, temperature, and incubation period, was performed by the variation of the pH range, temperature, and optical density of cell mass in which the maximum yield of the polymer was obtained.

Extraction of PHA

Hypochlorite Method. The copolymer was extracted from the bacterial cell with the chloroform hypochlorite method.[28] After 96 h of incubation of the culture medium, 10 mL of each microbial culture was centrifuged for 10 min at 6000 rpm. The pellet was collected and washed with 10 mL of saline solution and then recentrifuged in the same way. Then, the pellet was then suspended in 5 mL of sodium hypochlorite (4% active chlorine) and digested at 37°C for 40 min with stirring; this was followed by extraction with hot chloroform and precipitation with methanol. The extracted polymers were recentrifuged and washed with 10 mL of cold diethyl ether to get the purified P(3-HB-*co*-3-HV) products. The polymers were then vacuum-dried for 48 h. The polymer yield was determined with the following formula:

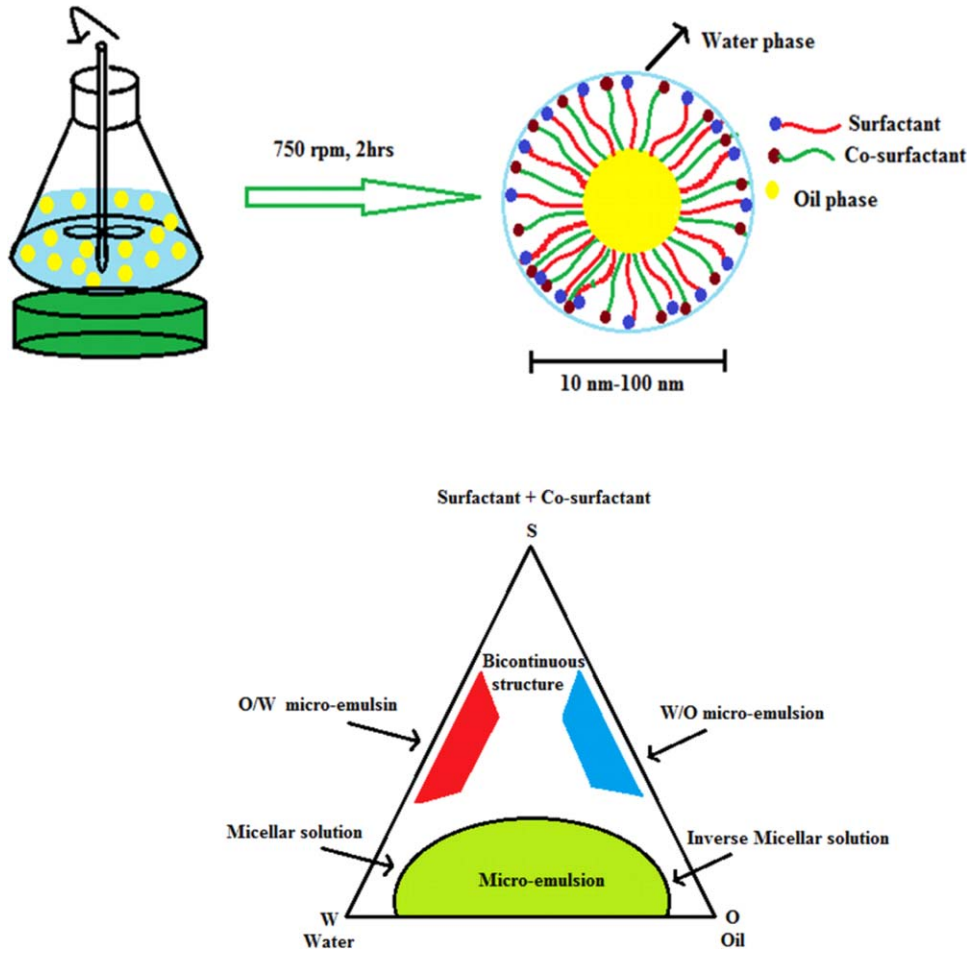


Figure 1. Schematic representation of an O/W microemulsion and the hypothetical phase region of the microemulsion. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$$S = \frac{P}{W} \times 100 \quad (1)$$

where S is the polymer yield (wt %), P is the polymer weight (g), and W is the dried cell mass (g).

Characterization

Globule Size Measurements of the O/W Microemulsion. The globule size of the microemulsion was determined by dynamic light scattering with a Malvern, Zetasizer Nano series, Nano ZS90, United Kingdom).

^{1}H-NMR Spectral Analysis. The chemical structure of the copolymer [P(3-HB-*co*-3-HV)] was confirmed by the measurement of the chemical shift position in ^{1}H-NMR spectroscopy. The analysis of the sample was carried out on a JEOL ALPHA-400 spectrometer. The 400-MH_Z ^{1}H-NMR spectra were recorded at 25°C in a $CDCl_3$ solution of the PHA copolymer (2 mg/mL). Tetramethylsilane was used an internal chemical shift standard.

Fourier Transform Infrared (FTIR) Spectral Analysis. FTIR spectroscopy was carried out with an attenuated total reflectance FTIR spectrometer (model Alpha, Bruker, Germany) with scanning from 4000 to 550 cm^{-1} for 42 consecutive scans at room temperature. The stretching frequency of each functional group (C—H, C—C, C—O, and C═O) was recorded with FTIR spectroscopy. Each polyester (1 mg) was mixed with 20 mg of KBr to form a KBr plate for IR analysis at 27°C.

Differential Scanning Calorimetry (DSC) Analysis. The thermal properties of the purified sample were investigated with DSC. For analysis, the sample (5 mg) was placed between two aluminum pans and compressed into a capsule. The capsule was placed in the DSC instrument, heated to within a temperature range of 30–200°C at a rate of 10°C/min, quickly cooled, and then scanned a second time over the same temperature range.

Gel Permeation Chromatography (GPC). The molecular weight of the polymer was determined by GPC. The system was furnished with a Styragel l 7.8 × 300 mm^2 column, one high performance liquid chromatography (HPLC) pump, and one refractive index detector (Waters). The mobile phase was chloroform. The flow rate was maintained at 0.3 mL/min. An about 4 mg of the PHA sample was dissolved in 2 mL of chloroform solvent with constant stirring at room temperature (30–32°C), and 80 µL of the sample solution was injected into the GPC column. For analysis, the 75-µL solution of polyesters was injected, and polystyrene was used as a molecular weight standard.[29] All of the data provided by the GPC systems were analyzed with Empower 2 software packages.

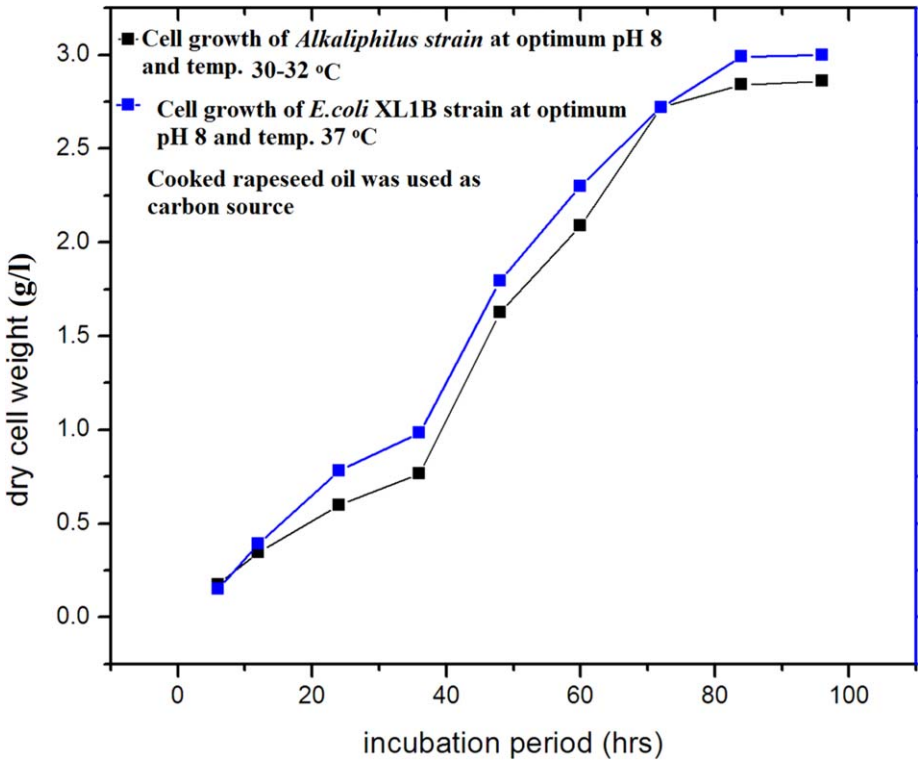


Figure 2. Effect of the incubation period (hours) on the microbial cell growth of the *A. oremlandii* strain and *E. coli* XL1B. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

X-ray Diffraction (XRD) Study. XRD measurement was recorded on a Rigaku RAD-111B instrument with nickel-filtered Cu Kα radiation (wavelength of 0.154 nm) at 40 kV and 30 mA. The X-ray analysis was performed at 27°C in the range 5–50° with a scanning speed of 20 min^{-1}.

RESULTS

Identification of the Isolated Strain

The isolated strain was then identified with the full-length sequence 16S rDNA technique and compared to those available in public databases. The results show that the isolated strain was closely related to the bacterial strain *A. oremlandii* OhILAs, and the allotted accession numbers for the 16S r DNA sequence was [NR_043674.1].

Recombinant *E. coli* XL1B Strain

The recombinant *E. coli* strain used was XL1-Blue (pSYL107), a genetically engineered, highly transformable strain. The stable high-copy number plasmid pSYL107 containing the *R. eutropha* PHA synthesis genes and the *E. coli ftsZ* gene were used intracellularly for the production of P(3-HB), as described previously.[24] The genotype of the strain was *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIq ZΔM15 Tn10 (Tetr)]*. The genes listed signifies mutant alleles of the XL1B strain. The XL-1 cells were tetracycline resistant, and the XL1-Blue cells were endonuclease (endA) deficient, which greatly improved the quality of miniprep DNA and were recombination (recA)-deficient; this improved the insertion stability. The hsdR mutation prevented the cleavage of cloned DNA by the EcoK endonuclease system. The *lacIq ZΔM15* gene on the F′ episome allowed blue–white color screening.

Measurement of the Dry Cell Weight and the Percentage of Polyester under Optimized Conditions

The growth of the bacterial cells was monitored at 600 nm for definite time periods with an ultraviolet–visible spectrophotometer. The growth of the density of each bacteria was recorded during the incubation period (Figure 2).

The variation of the dry cell weight was calculated, and the percentage of biopolymer accumulation of each of the microbial strains is shown in Table I. With increasing incubation period, the cell density or dry cell weight increased. The dry cell weight and percentage of intracellular polyesters were also changed

Table I. Extent of Biopolymer Accumulation by the *A. oremlandii* and *E. coli* XL1B Strains After a 96-h Incubation with Rapeseed Oil as the Sole Carbon Source at a Variety of Temperatures and at an Optimum pH of 8

Temperature (°C)	PHA accumulation by the *A. oremlandii* strain (%)	PHA accumulation by the *E. coli* XL1B strain (%)
25	41	—
28	68	—
31	94	38.33
34	78	—
37	48	62.45
43	—	41.15

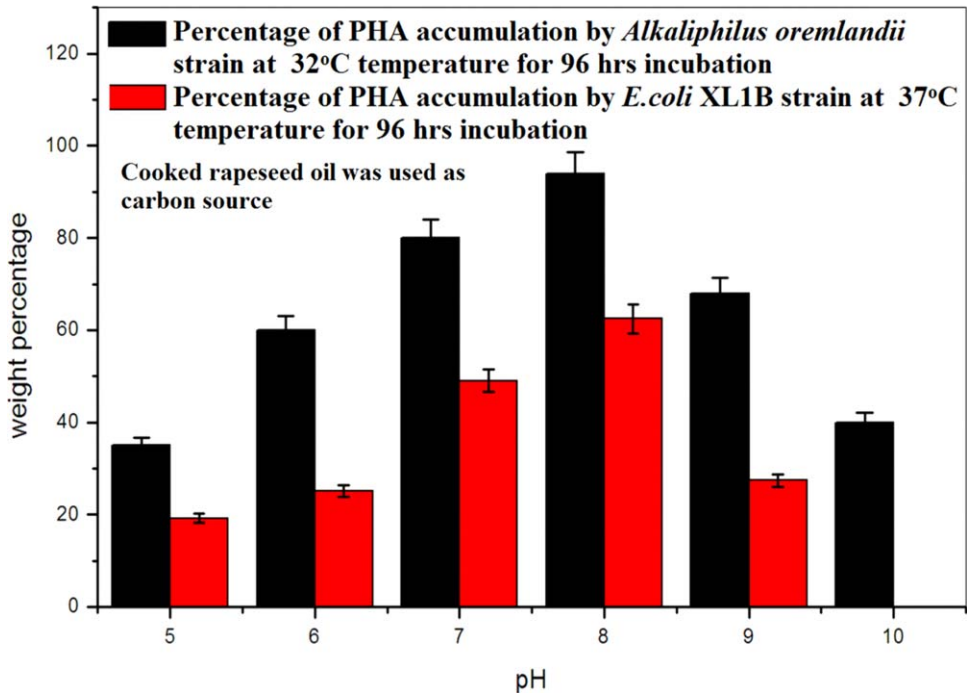


Figure 3. Effect of the pH on intracellular PHAs by the *A. oremlandii* strain and the *E. coli* XL1B strain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with the variation of the culturing conditions, such as the pH of the medium, incubation periods, inoculum density, temperature effect, and concentration of cooking oil in the culture medium. The intracellular accumulation capacity of the polyesters also depended on the type of enzymes involved in the synthesis process. The growth conditions of the *Alkaliphilus* strain differed from those of the *E. coli* and *B. cereus* strains. The variation of the percentage of polymer accumulation was found to be dependent on the source of the cooking oil. In the case of the *Alkaliphilus* strain, to identify the optimum parameters (pH, incubation time, temperature, and quantity of the carbon source) responsible for the accumulation of the maximum yield of the polymer, a number of sets of experiments was performed with the variation of pH and temperature range with cooked rapeseed oil as the carbon source. The optimum pH value for the maximum accumulation of the biopolymer was found at

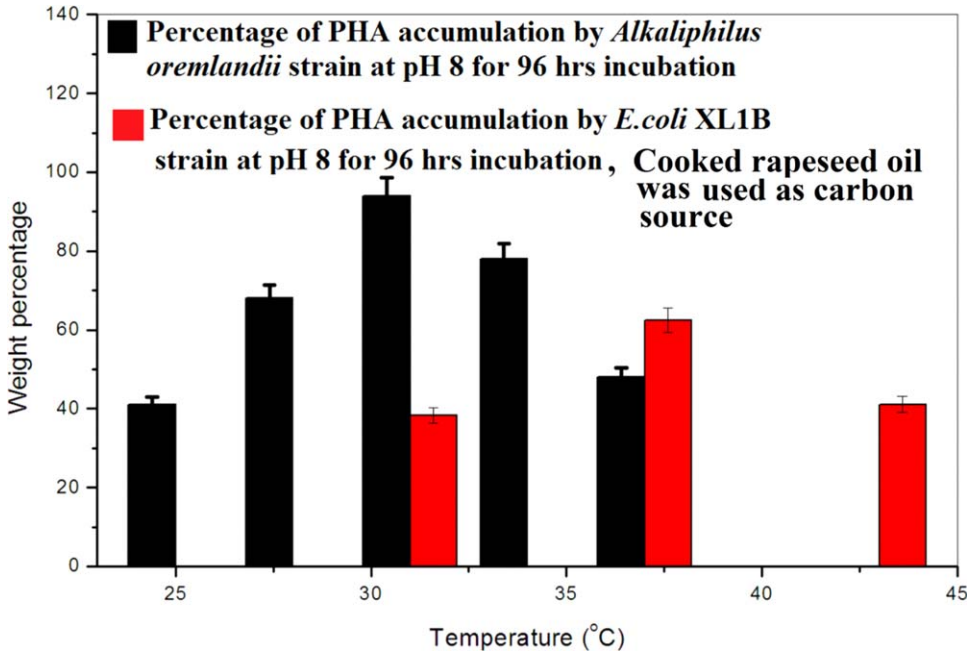


Figure 4. Effect of the temperature on the production of bacterial PHAs by the *A. oremlandii* strain and the *E. coli* XL1B strain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table II. Comparison of PHA Production by the *A. oremlandii* Strain and the Control *B. cereus* Strain under Optimum Conditions (Incubation Time, pH, and Temperature) with a Variety of Different Spent Oils

	A. oremlandii bacterial strain, optimum conditions of pH 8 and 30–32°C, and 96-h incubation		*B. cereus* bacterial strain, optimum conditions of pH 7.5 and 30°C, and 48-h incubation	
Food source	PHA accumulation (%)	3-HV unit (mol %)	PHA accumulation (%)	3-HV unit (mol %)
Soybean oil	95.3	16.04	59.09	19.59
Rapeseed oil	94	24.95	52	15.07
Mustard oil	93.15	16.3	47.7	19.24
Palm oil	94.57	25.62	49.85	13.84
Coconut oil	85.78	16.52	41.54	17.27

pH 8, and in extreme acidic and alkali media, the growth of cell density and the polymer accumulation capability were inhibited, as shown in Figure 3. Experiments with the *A. oremlandii* strain over a wide range of temperatures (25–37°C) showed that the accumulation of PHA increased up to 32°C, in which a maximum yield of biopolymer was obtained in the stationary phase, and then, it decreased (Figure 4). Through the application of the optimum conditions, the PHA production was also investigated with different cooking oils (soya oil, mustard oil, coconut oil, rapeseed oil, and palm oil). The maximum yield of the biopolymer was found for soya oil (95.3%), as shown in Table II. The results obtained by the culturing of the *Alkaliphilus* strain with different cooking oils were also compared with those of the well-known bacterial strain *B. cereus,* and we found that the maximum percentage of biopolymer accumulation was obtained for the *Alkaliphilus* strain, as shown in Figure 5 and Table II. However, the intracellular accumulation of the biopolymer by the *E. coli* strain was 62.45% on the basis of the dry cell weight

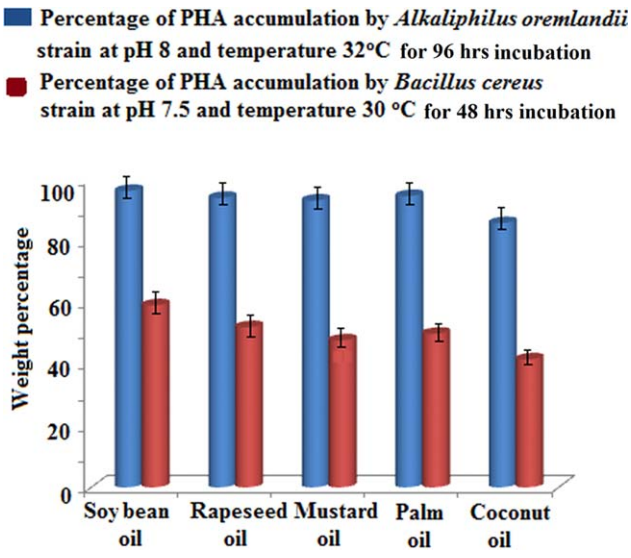


Figure 5. Percentage of the accumulation of PHA by the *A. oremlandii* strain and the *B. cereus* strain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

at 37°C when the pH and incubation time were the same as in the growth conditions for the *Alkaliphilus* strain (Table III).

FTIR Spectral Analysis

In the FTIR spectrum of P(3-HB-*co*-3-HV) [Figure 6(II)], the peaks at 2930, 1727, and 3429 cm^{-1} are the characteristics peaks of methine (—CH), carbonyl (C═O), and hydroxyl (—OH) groups, respectively. The methyl ($—CH_3$) group gave a strong band in range 1383–1449 cm^{-1} because of the stretching and bending mode of vibration of that functional group. The absorption peak at near 1283 cm^{-1} was associated with the saturated ester linkage of C—O groups in the biopolymer.

On the other hand, the FTIR spectra [Figure 6(I)] of the *E. coli* based polyester showed an absorption band at 1743 cm^{-1} due to the ester carbonyl group. The bands at 2921, 1454, 1161, and 3384 cm^{-1} were the characteristic peaks of the asymmetric stretching vibrations of methyl protons, bending mode of the vibration of methylene protons, asymmetric stretching vibrations of the C—O—C linkage, and —OH functional groups (with a strong, broad-spectrum peak), respectively.

^{1}H-NMR Spectral Analysis

As shown in Figure 7(I), ^{1}H-NMR spectrum of polyester prepared by the *Alkaliphilus* strain indicated the presence of

Table III. Extent of Biopolymer Accumulation by the *A. oremlandii* Strain at the Optimum Temperature of 32°C and by the *E. coli* XL1B Strain at the Optimum Temperature of 37°C After a 96-h Incubation with Rapeseed Oil as the Sole Carbon Source with Variations in the pH

pH	PHA accumulation by the *A. oremlandii* strain (%)	PHA accumulation by the *E. coli* XL1B strain (%)
5	35	19.2
6	60	25.13
7	80	49
8	94	62.45
9	68	27.43
10	40	—

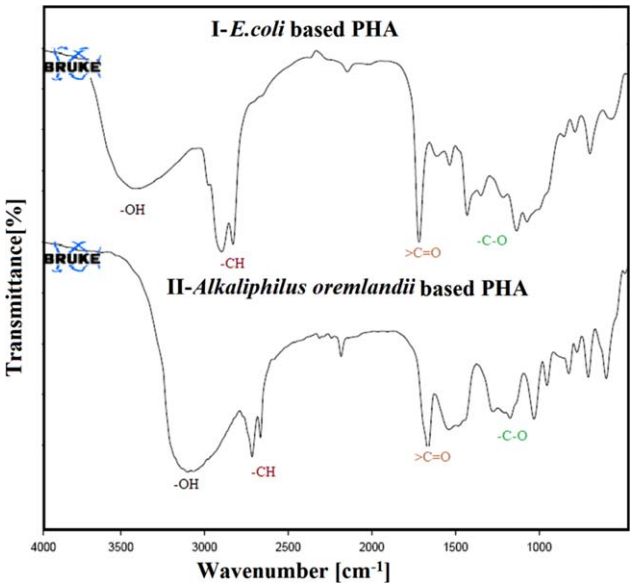


Figure 6. FTIR spectra of (I) P(3-HB) and (II) P(3-HB-*co*-3-HV) obtained with the *A. oremlandii* strain and the *E. coli* XL1B strain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

different chemical shift values: $\delta_1 = 1.253$ ppm (—CH_3, 3-HB group), $\delta_2 = 0.878$ ppm (—CH_3, 3-HV gr.), $\delta_3 = 1.606$ ppm (—CH_2, 3-HV gr.), $\delta_4 = 2.307$ ppm (—CH_2, 3-HB—3-HV gr.), and $\delta_5 = 5.340$ ppm (—CH_2, 3-HB—3-HV gr.).

Again, the ^{1}H-NMR spectra [Figure 7(II)] of the polyester obtained by the *E. coli* strain showed major peaks at $\delta'_1 = 1.25$ ppm (—CH_3), $\delta'_2 = 2.2$ ppm (—CH_2), and $\delta'_3 = 5.21$ ppm (—CH), and these were due to the resonance absorption spectra of methyl group protons (—CH_3).

XRD Analysis

The XRD spectra of the pure PHAs were separated out from the bacterial cells of the *Alkaliphilus* and *E. coli* strains, as shown in Figure 8(I,II). The pure polyesters showed six peaks at about 13.2, 16.17, 21.14, 27.5, 31.9, and 45.6°, in which the main peaks at 13.2 and 16.17° were observed as the characteristic peaks of the biopolymer with the corresponding Millar indices of (110) and (020). The XRD analysis of both the homopolymer and copolymer indicate nearly similar values of glancing angle but a broadening of peak intensity for the copolymer; this indicated the presence of the semicrystalline nature of the polyester.[30]

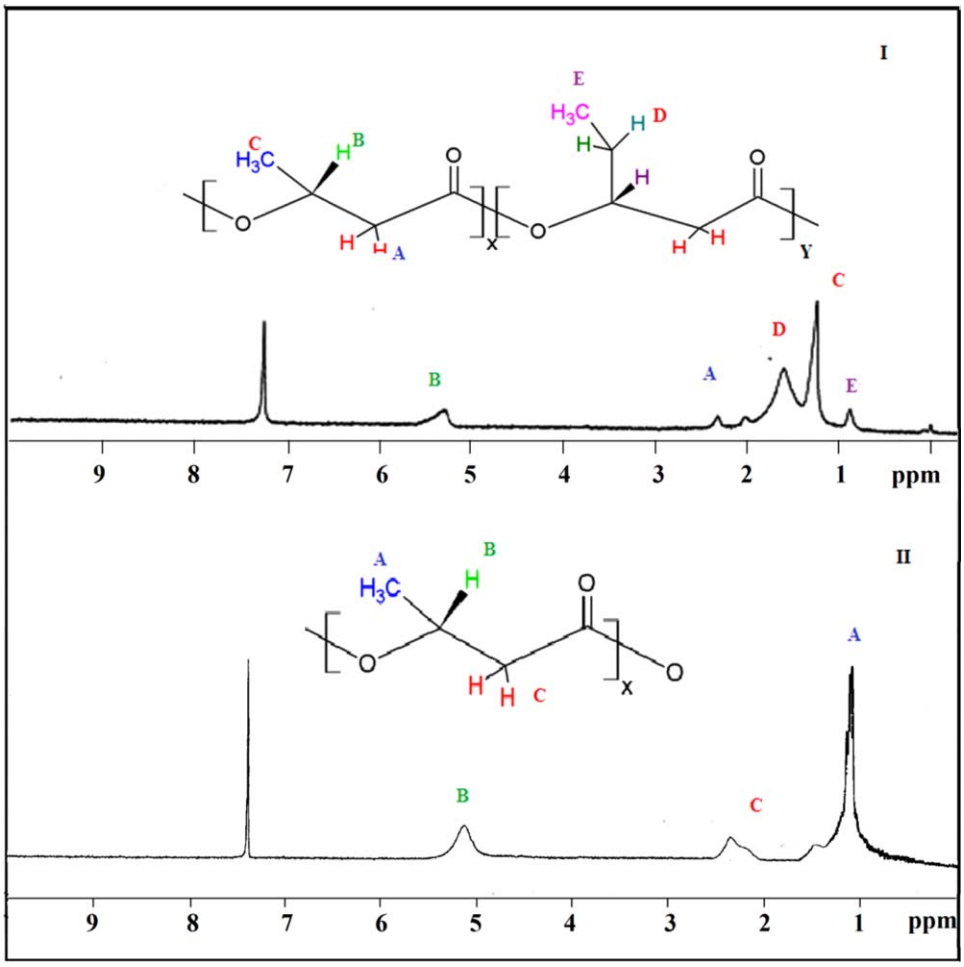


Figure 7. ^{1}H-NMR spectra of (I) P(3-HB-*co*-3-HV) and (II) P(3-HB) obtained with the *A. oremlandii* strain and the *E. coli* XL1B strain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

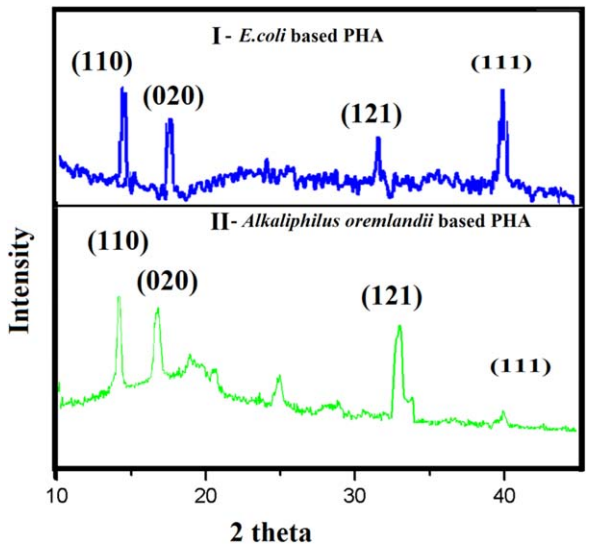


Figure 8. DSC characterization of PHAs [(I) P(3-HB) and (II) P(3-HB-*co*-3-HV)] extracted from the *A. oremlandii* strain and the *E. coli* XL1B strain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

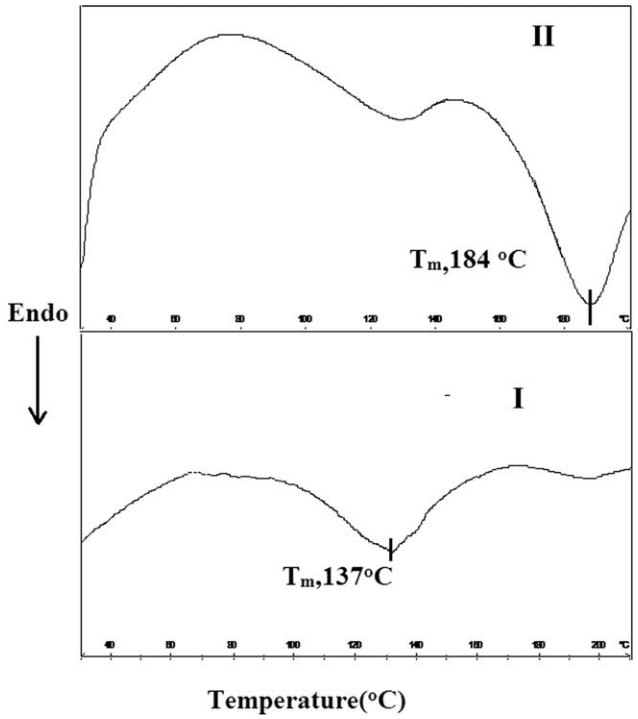


Figure 9. XRD patterns of (I) P(3-HB-*co*-3-HV) and (II) P(3-HB) extracted from bacterial cells.

GPC Analysis

From the GPC data for the determination of the weight-average molecular weight (M_w) of polyesters, it was shown that the M_w of the copolymer was lower (2.2×10^5 g/mol) than that of the standard P(3-HB) homopolymer but higher than the molecular weight of the polyester [36.3 KDa] obtained from the oil source reported by Haba et al.[21] The number-average molecular weight (M_n) and polydispersity index (PDI = M_w/M_n) of the *Alkaliphilus* strain based biopolymer were 1.235×10^5 Da and 1.73, respectively.

On the other hand, the M_w of P(3-HB) obtained by the *E. coli* strain was 3.2×10^6 Da but was nearly similar to the molecular weight reported by Sudesh.[31]

DSC Analysis

The T_m, heat of fusion (ΔH_f), and percentage X_c of the biopolyesters synthesized by the *Alkaliphilus* and *E. coli* strains are reported in Table IV and shown in Figure 9(I,II). The T_m and X_c values of these polyesters were observed to be lower than those of the standard 9:1 P(3-HB-*co*-3-HV) (143°C).[32] The sharp decrease in the T_m of the polyester was due to the increase in the molar fraction of the 3-HV unit in the polymer chain.[33]

On the other hand, the *E. coli* based biopolymer showed a T_m of 184°C. The high value of T_m was nearly equal to the T_m of the standard P(3-HB) (177°C)[28] and was much higher than that of the standard 9:1 P(3-HB-*co*-3-HV) (143°C).

The X_c of a polymer is temperature-dependent and is calculated with the following equation:

$$X_c = \Delta H_f(T_m)/\Delta H_f^o\ (T_m^o)$$

where $\Delta H_f(T_m)$ is the enthalpy of fusion measured at the melting point and $\Delta H_f^o(T_m^o)$ is the enthalpy of fusion of the totally crystalline polymer measured at the equilibrium melting point (T^o_m).

Table IV. Thermal Properties of the P(3-HA-*co*-3-HV) Copolymer and the PHB Homopolymer Produced by the *A. oremlandii* Strain and the *E. coli* XL1B Strain

Polymer source	Polymer	M_w (g/mol)	PDI	T_m (°C)	ΔH_f (cal/g)	X_c
Sigma-Aldrich, Kurkarni et al.[32]	Standard P(3-HB-co-3-HV) sample	—	—	143.79	13.32	38.31
Rapeseed oil, pH 8, 32°C, optimum conditions (96-h incubation)	P(3-HB-co-3-HV) sample	2.2×10^5	1.7	137	7.6	22.32
Sigma- Aldrich, Kurkarni et al.[32]	Standard P(3-HB) sample	—	—	177.24	24.97	71.83
Rapeseed oil, pH 8, 37°C, optimum conditions (96-h incubation)	P(3-HB) sample	3.2×10^6	1.9	184	26.12	75.13

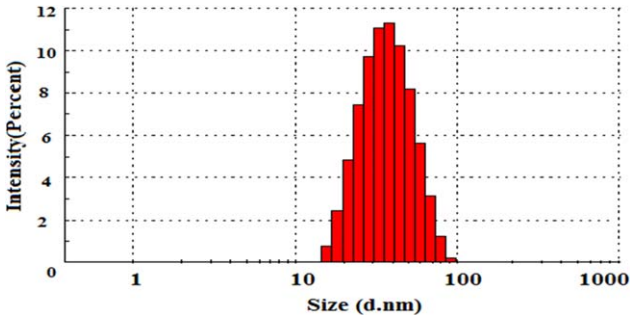


Figure 10. Characterization of the globule size in the O/W microemulsions by dynamic light-scattering analysis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The X_c of the *E. coli* based polyester (75.13%) was higher than that of the standard P(3-HB) (71.83%), but the X_c of the 25% 3-HV unit content *Alkaliphilus* based polyester was 22.32%; this was lower than that of the standard 9:1 P(3-HB-*co*-3-HV) (38.31%).[30] The decrease in the X_c of the copolymer was due to the incorporation of 3-HV units.[34]

DISCUSSION

The O/W microemulsion was prepared to reduce the interfacial tension between oil and water and also to enhance the biological contact area of the globule by decreasing the droplet size. The globular size in the O/W microemulsion was analyzed, and the average particle size was found to be nearly 42 nm, as shown in Figure 10. The PDI value of the transparent solution was found to be 0.4961 below 1.00; this indicated the long-term stability of the microemulsion system.

The growth of bacterial cells depends on the culturing conditions, including the temperature, acidity, and alkalinity, and also on components present in the sterilized medium. From the experimental analysis, we observed that the growth of the bacterial cell density was too much low (0.152 mg/mL) in the initial stage. It took 24 h to reach the stage of sufficient cell growth (0.780 mg/mL), after which the cell density rapidly increased to 2.3009 mg/mL within 60 h of incubation because of the increase in metabolic activity and the DNA replication by binary fission at a constant rate. In the last step of the kinetics of bacterial growth, that is, in stationary phase, the cell number did not increase with elapsed time, and thus, the growth rate was stabilized with a saturated optical density that was equivalent to a concentration of 2.86–3.00 mg/mL. Bacterial cultures with both the *A. oremlandii* and *E. coli* XL1B strains in the microemulsion kinetically showed nearly the same growth rate. The growth of the cell density and the extent of accumulation (the percentage) of intracellular PHA in the emulsified medium showed higher values compared to earlier reported ones.[28,29]

The cell density of the bacterial strains in the growth media was investigated in media at different pHs (5, 6, 7, 8, and 9), as shown in Table I. The cell density increased sharply with increasing pH from 5 to 8, and then, it dropped (Figure 3). The maximum cell growth and biopolymer production for the *Alkaliphilus* strain and for the *E. coli* strain were facilitated at an alkaline pH of 8. The polymer production efficiency of the *Alkaliphilus* strain was compared to that of the control bacterial strain *B. cereus* with different cooking oil sources (soya oil, rapeseed oil, coconut oil, palm oil, and mustard oil). The results shown in Figure 5 and Table II prove that the polymer accumulation capacity was always greater for the *Alkaliphilus* strain for different cooking oil sources, and the maximum yield of the biopolymer was 95.3% on the basis of dry cell weight for soya oil. Like the *Alkaliphilus* strain, the intracellular synthesis of P(3-HB-*co*-3-HV) from a simple carbon source was performed with the moderately haloalkali tolerant *Halomonas campisalis* MCM B-1027 at an alkaline[32] pH of 9, but the yield was 58%; this was comparatively lower than the 94–95% yield obtained with the *Alkaliphilus* strain. The optimum pH (8) for the maximum biopolymer accumulation by the *E. coli* XLIB strain was found to be similar to that of the P(3-HB-*co*-3-HV) production by *R. eutropha*.[35] Again, the yield of P(3-HB-*co*-3-HV) obtained by the well-known bacterial strain *B. cereus* with the different cooking oils (soya oil, palm oil, coconut oil, rapeseed oil, and mustard oil) was also comparatively lower than the yield obtained by the *Alkaliphilus* strain, as shown in Table II.

The temperature effect is one of the most important factors influencing bacterial cell growth. A prominent relationship between the square root of cell growth and the temperature was proposed by Ratkowsky et al.,[36] in which specific ranges of temperature were well-defined for bacterial growth. At lower ranges of temperature, the metabolic activity of enzymes was not observed. At higher ranges of temperature, a heat shock protein was released, and denatured proteins aggregated and became nonfunctional because of the increased stress of temperature, and thus, cell growth was prevented.[37] Therefore, from the experimental results, we concluded that the maximum accumulation of the biopolymer by the *A. oremlandii* strain was only possible in a certain range of optimized temperature (30–32°C). However, for the mesophilic bacterial strains *E. coli* and *B. cereus*, the maximum cell densities were observed at 37 and 30°C, respectively. The experimental analysis was comparable to that of Nguyen.[38] In this experiment, a maximum yield of 62.45% of P(3-HB) was obtained at 37°C. The yield of the homopolymer in lower and higher molecular weight ranges was found to be low, as shown in Table III.

The ^{1}H-NMR spectrum of the polyester obtained by the *Alkaliphilus* strain indicated that the methyl protons (—CH_3) of the 3-HB side group had a doublet resonance at 1.253 ppm and the methyl protons (—CH_3) of the 3-HV side group resonated at 0.878 ppm with a triplet resonance because of the coupling with the adjacent methylene group. A group of strong absorption peaks at 1.606 and 2.307 ppm for the ethylene protons (—CH_2) of the 3-HV side groups and —CH_2 of the 3-HV–3-HB bulk structure with multiple resonances, respectively; this indicated the splitting of adjacent methylene and methyl protons in the presence of an external magnetic field. Again, the methine proton (—CH, 3-HV and 3-HB bulk structures) linked with more electronegative oxygen atoms had a multiplet resonance with a more downfield chemical shift value at 5.340 ppm because of the deshielding effect of the methine proton. From the data

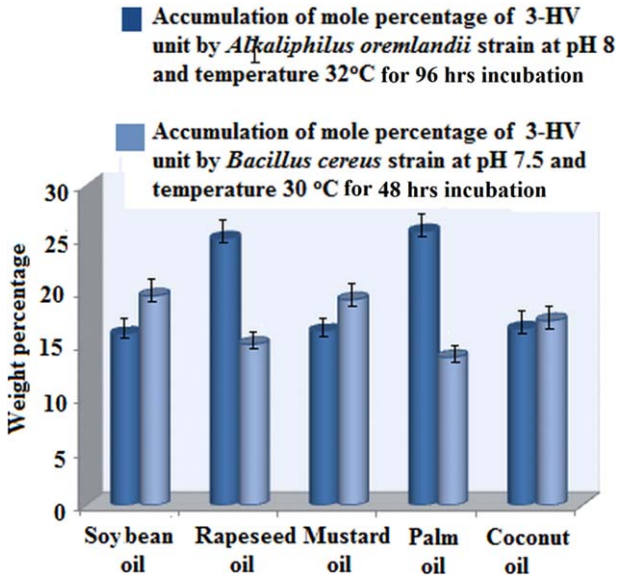


Figure 11. Percentage of the accumulation of 3-HV units by the *A. oremlandii* strain and the *B. cereus* strain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

analysis, we concluded that the polyester formed from the *Alkaliphilus* strain was a copolyester of P(3-HB-*co*-3-HV). These results were comparable to those of a previous report.[39] The molar percentage of the 3-HB unit and the 3-HV unit in the copolymer were calculated from the intensity ratio of the methyl groups for the 3-HB and 3-HV groups according to a method described by Bloembergen and Holden.[40] The percentage molar fraction of the 3-HV unit was calculated for both the *Alkaliphilus* strain and control *B. cereus* and is reported in Figure 11 and Table II. The maximum molar percentage of 3-HV units was obtained from waste palm oil (25.62%) by the culturing of the *Alkaliphilus* strain, and in case of *B. cereus*, it was only 19.59% for waste soya oil.

Again, the ^{1}H-NMR spectra of the polyester obtained by the *E. coli* strain showed major peaks at 1.25, 2.2, and 5.21 ppm, and these were due to the resonance absorption spectra of methyl group protons (—CH_3), methylene group protons (—CH_2), and methane group proton (—CH), respectively. These chemical shift values indicated the presence of only 3-HB units in the polyester. Therefore, the ^{1}H-NMR spectral analysis of the polyesters obtained by the *Alkaliphilus* strain and *E. coli* strain indicated that the *Alkaliphilus* strain was capable of accumulating the P(3-HB-*co*-3-HV) copolymer directly from the oil source, but *E. coli* was able to synthesize only the P(3-HB) homopolymer in its cytoplasm.

As shown in Figure 6(II), a strong band at 1283 cm^{-1} indicated ester bonding in the copolymer. The methyl group (—CH_3) provided a peak at 1383 cm^{-1}, whereas the methylene groups (—CH_2) at 1449 cm^{-1} were due to bending mode vibrations. The methine group (—CH) at 2930 cm^{-1} was due to asymmetric stretching vibrations, and the peak of the —C═O functional group at 1727 $cm^{-1}$and the strong absorption peak of the hydroxyl group (—OH) at 3429 cm^{-1} confirmed the chemical structure of the copolymer. The FTIR analysis of the copolymer was completely in agreement with the results of earlier reports.[41–43] On the other hand, the FTIR spectra [Figure 6(I)] of the *E. coli* based polyester showed the absorption band at 1743 cm^{-1} due to the ester carbonyl group. The bands at 2921, 1454, 1161, and 3384 cm^{-1} were the characteristic peaks of the asymmetric stretching vibrations of methyl protons, the bending mode vibrations of methylene protons, the asymmetric stretching vibrations of the C—O—C linkage, and the —OH functional groups (with a strong, broad-spectrum peak), respectively.

The X_c of the polyester depends on the composition involved in the structure of the polymer chain. The X_c of the P(3-HB-*co*-3-HV) copolymer decreased as the *d*-spacing value of the Millar indices (110) increased because of steric effects generated by an ethyl group of 3-HV units, and a nearly orthorhombic crystal of P(3-HB) was found.[34] The X_c of the biopolymer decreased with decreasing chain length and an increase in different branching monomer units.[42] As shown in Figure 8(I), the crystalline nature of the *E. coli* based polyester was maximum, and the sharp intense peaks at 13.2 and 16.17° were due to the presence of the pure homopolymer. However, the X_c of the copolymer isolated from the *Alkaliphilus* strain [Figure 8(II)] decreased; this might have been due to the increase in the molar fraction of 3-HV units[33] in the polymer chain throughout the incubation period. X_c was found to be the lowest when the polymer contained the maximum concentration of 3-HV units (i.e., a 25.3% molar fraction of 3-HV units when cooked palm oil was used as the sole carbon source).

The thermal properties of a biopolymer mainly depend on its T_m, enthalpy change, and X_c. The variation of X_c and T_m of the biopolymer is due to the presence of different percentage of comonomers in the polymer skeleton. The T_m of the *E. coli* based biopolymer was found to 184°C, and the *Alkaliphilus* strain based biopolymer showed a T_m of 137°C. The sharp decrease in T_m of the polyester was due to the increase in the molar fraction of 3-HV units in the polymer chain.[34] The comparatively higher X_c of the homopolymer versus that of the copolymer was due the crowding effect of the bulk valerate units in the polymer backbone.[33]

CONCLUSIONS

The synthesis of a P(3-HB-*co*-3-HV) copolymer with different concentrations of 3-HV units (16.04, 16.3, 16.52, 24.95, and 25.62%) was performed by the *A. oremlandii* strain with different waste cooking oils (soya oil, mustard oil, coconut oil, rapeseed oil, and palm oil), whereas only P(3-HB) was obtained by the *E. coli* strain in a common growth medium after a 96-h incubation period. Different contents of 3-HV units in the PHAs obtained for different cooked oils by the *Alkaliphilus* strain were compared with the results obtained by the *B. cereus* strain, and we found that the contents of 3-HV units in PHAs and the copolymer accumulated from 2% waste cooking oil was much higher than those of the copolymer and homopolymer isolated by the respective well-known bacterial strain *B. cereus* (optimum pH 7, temperature = 30°C) and *E. coli* XL1B strain (optimum pH 8, temperature range = 25–43°C]. The high yield of the polymer in the O/W microemulsion was due to the

increase in contact area for the accumulation of nutrients in the bacterial cell body; this reinforced the growth of the cell mass and polymer formation via an active enzymatic intermediate. Therefore, the synthesis of copolymers with lower X_c values from toxic waste cooking oil may find applications in the area of biomedical engineering.

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