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ORIGINAL PAPER

Biosynthesis and characterization of copolymer poly (3HB-*co*-3HV) from saponified *Jatropha curcas* oil by *Pseudomonas oleovorans*

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Abstract Polyhydroxyalkanoates (PHAs) are naturally occurring biodegradable polymers with promising application in the formulation of plastic materials. PHAs are produced by numerous bacteria as energy/carbon storage materials from various substrates, including sugars and plant oils. Since these substrates compete as food sources, their use as raw material for industrial-scale production of PHA is limited. Therefore, efforts have been focused on seeking alternative sources for bacterial production of PHA. One substrate that seems to have great potential is the seed oil of *Jatropha curcas* plant. Among other favorable properties, *J. curcas* seed oil is non-edible, widely available, and can be cheaply produced. In this study, *Pseudomonas oleovorans* (ATCC 29347) was grown in a mineral salt medium supplemented with saponified *J. curcas* seed oil as the only carbon source under batch fermentation. Optimum PHA yield of 26.06% cell dry weight was achieved after 72 h. The PHA had a melting point (T_m) between 150 and 160°C. Results of polymer analyses by gas chromatography/mass spectrometry (GC/MS) identified only the methyl 3-hydroxybutanoate monomeric unit. However, electrospray ionization–time of flight mass spectroscopy (ESI–TOF MS) confirmed that the PHA was a copolymer with the characteristic HB/HV peaks at *m*/*z* 1155.49 (HB) and 1,169, 1,184–1,194 (HV). The data were further supported by ^{1}H and ^{13}C NMR analysis. Polymer analysis by gel permeation chromatography (GPC) indicated a peak molecular weight (MP) of 179,797, molecular weight (M_W) of 166,838, weight number average mass (M_n) of 131,847, and polydispersity (M_w/M_n) of 1.3. The data from this study indicate that *J. curcas* seed oil can be used as a substrate to produce the copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), poly(3HB-*co*-3HV).

Keywords *Jatropha curcas* · Polyhydroxyalkanoate · Copolymer · Biodegradable · *Pseudomonas oleovorans* ATCC 29347

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Introduction

Synthetic plastics are well known to be generally non-biodegradable. They accumulate in the environment at a rate of several million tons per year. When burnt, they produce greenhouse gasses, such as hydrogen cyanide and hydrogen chloride and are aesthetically unpleasant [21, 38]. Thus, there is an urgent need to seek alternative sources for biodegradable and biocompatible polymers. One major source that has gained attention in recent years is a class of natural polyester commonly known as polyhydroxyalkanoates (PHAs) [44]. PHAs consist of about 120 different (*R*)-3-hydroxyacid (3HA) monomeric units with the most common being poly(*β*-hydroxybutyrate)(PHB) [19] and poly(*β*-hydroxyvalerate) (PHV) [5]. Several bacteria use various carbon substrates to produce PHAs under limiting growth conditions [1, 13, 24, 26–28, 50]. *Pseudomonas oleovorans*, for example, employs substrates requiring *β*-oxidation, but not those requiring fatty acid de novo synthesis to produce medium chain length (mcl) PHAs [11, 12, 14, 17, 23, 36, 43, 49, 51]. Brand et al. reported the synthesis of mcl-PHAs by *P. oleovorans* using *n*-alkanes (C_8 to C_{12}) [25] and *n*-alkanoic acids as substrates [4]. One

challenge to the wide industrial application of these and other substrates as feedstock is their cost. They are expensive and may compete with other applications. It is estimated that feed stock accounts for about 30% of PHA net production [40]. Therefore, there is need to minimize cost by seeking cheap and renewable sources of carbon substrates [8, 10]. Plant oils seem to offer a more attractive alternative since they contain a greater number of carbon atoms and produce PHA with varied alkyl chain length and higher molecular masses. Plant oils can generate a theoretical yield coefficient of 0.65–0.98 g PHA/g when compared to glucose, which only generates 0.32–0.48 g [7, 20]. Numerous examples exist of PHA production from plant oils; these include soybean, castor, euphorbia, vernonia, and other vegetable oils [2, 10, 17, 20, 39, 47]. However, the consumable nature of these oils limits their large-scale industrial usage for PHA synthesis.

One plant oil that has not been well studied for its potential use in PHA production is the *Jatropha curcas* seed oil. It is non-consumable, has low acidity, low viscosity, good oxidation stability, cold properties, and can be cheaply produced [3, 18, 31, 35, 37, 48]. *J. curcas* plant belongs to the genus *Euphorbiaceae*. It is native to South/Central America but grows ubiquitously in most tropical regions of the world. It is not grazed, highly drought resistant, and is adapted to diverse conditions [15]. The major fatty acids (FA) in the oil are oleic (18:1), 41.5 to 48.8%; linoleic [18:2], 34.6–44.4%; and palmitic (16:0), 10.5–13.0% [33]. Despite the many desirable properties and plethora of potential applications of *J. curcas* seed oil, its use as a source of PHA biosynthesis is not known. Therefore, here we explore the potential of *J. curcas* seed oil in the biosynthesis of PHA by *Pseudomonas oleovorans* (ATCC 29347).

Materials and methods

Microbial culture

Pseudomonas oleovorans (ATCC 29347) was obtained from the American Type Culture Collection (ATCC) and subcultured on trypticase soy broth (Becton Dickinson) and King's medium. Stock cultures were maintained on trypticase soy agar (Becton Dickinson) at 5°C. The level of inoculum for all batch fermentation was 5 mL of 2×10^8 cfu/mL *P. oleovorans*. All media were prepared as described by the manufacturer.

Saponification of *Jatropha curcas* oil

Saponification of oil was done by refluxing 100 mL methanol (Fisher), 4.95 g sodium hydroxide, and 10.0 g *Jatropha curcas* oil for 30 min. The hot mixture was added to 50 g ice and 50 g water. The resulting semisolid was crushed, filtered, and dried. The percentage yield was 30% based on linoleic acid. Saponification was repeated several times under before mentioned conditions.

Mineral salt medium (MSM)

The mineral salt medium (MSM) used for PHA production was prepared as described elsewhere [2] and contained saponified *Jatropha curcas* oil (0.98 g/L) as the sole carbon source. The medium was sterilized for 15 min at 121°C before use.

Batch fermentation

A culture flask was charged with 2,500 mL MSM and 5.0 mL of 2×10^8 cfu/mL *P. oleovorans* (ATCC 29347). Incubation was done at 25°C and 120 rpm on a Lab-line Environ incubator (USA) and a gyrotory shaker (New Brunswick, NJ, USA). Fermentation was stopped at 72 h. Cells were harvested by centrifugation, lyophilized, and weighed. The polymer was extracted and analyzed. Batch fermentation was repeated five times under previous conditions.

Batch fermentation under varying temperatures and pH

The effect of temperatures 25, 30, and 37°C, and initial pH 5, 6, 7, 8, and 9 on PHA yield was investigated. Batch fermentation was done as mentioned previously. Temperatures were maintained by using a Lab-line Environ incubator at 120 rpm. The initial pH for the fermentations was derived by adjusting the mineral salt medium with dilute hydrochloric acid (HCl). Furthermore, initial pH was not controlled during fermentations and fermentations were repeated five times.

Isolation of biomass (dry cell mass)

Fermentation broths were centrifuged for 15 min, 13,000 rpm, and 4°C in a Sorvall RC-5 refrigerated superspeed centrifuge (Dupont Instruments, Newtown, CT, USA). The resulting pellet was resuspended by washing once with 20 mL Tris–HCl buffer (pH 7.2) and centrifuged. Pellets were shelled by using a mixture of dry ice and acetone (Fisher), and lyophilized by using a LABCONCO Freeze Dryer 5 instrument (Kansas City, Missouri), with settings at −50°C and 25 mmHg.

Extraction and purification of PHA

One hundred milligrams of lyophilized cells was added to 100.0 mL hot chloroform (Fisher, HPLC grade) and refluxed

for 3 h. The hot mixture was filtered through a Whatman cellulose extraction thimble (43 × 123 mm single thickness, Aldrich, Milwaukee, WI, USA) and the solvent was allowed to evaporate freely at room temperature. The resulting oily film was purified by repeatedly precipitating/dissolving the polymer with methanol and chloroform respectively. The final precipitate was dried and stored until further analyses.

Melting point (T_m) determination of polymer

Melting point was determined by sandwiching 0.01 mg of polymer between two cover glasses and placed onto the heating table of a Fisher Johns melting point apparatus (Fisher Scientific Company, USA), equipped with a −10 to 260°C PG Extco alcohol thermometer. Samples were analyzed in triplicate.

Analyses of PHA

The purified material was analyzed by using gas chromatography/mass spectrometry (GC/MS), electrospray ionization–time of flight mass spectrometry (ESI–TOF MS), proton and carbon (^{1}H and ^{13}C) nuclear magnetic resonance (NMR) spectroscopy, and gel permeation chromatography (GPC).

Gas chromatography/mass spectrometry (GC/MS)

Samples were base transesterified to their monomers as outlined elsewhere [2]. An Agilent Technologies 6890 N Network GC System (CA, USA) interfaced directly to an Agilent Technologies 5973 Inert Mass Selective Detector was used to generate data. A Supelco fused-silica SPB-1 (30 m, 0.32-mm i.d., 0.25-μm film) column (Bellefonte, PA, USA) was used to conduct high-resolution capillary gas chromatography. Oven temperature was programmed from 40 to 300°C at 12°C/min, and helium was used as the carrier gas with head pressure of 3 psi. Injector temperature was set at 240°C. About 1.0 μL of the transesterified sample was injected into the GC/MS instrument and the run started. A Hewlett-Packard PC integration program was used to calculate the peak areas and percentages of the monomeric components of the polymer. Runs were done in triplicate.

Electrospray ionization–time of flight mass spectrometry (ESI–TOF MS)

Samples were partially base transesterified as before and infused at 30.0 μL/min. The following instrument (Agilent 6210 TOF LC/MS) specification was used in the MS program: dry gas, 12.0 L/min; dry temperature, 350°C; nebulizer, 50 psi; scan, 50–1,000 at 40 Hz; fragmentor, 200 V; skimmer, 60 V; capillary, 5,000 V. A ZORBAX SB C18, 2.1 × 50 mm, 1.8-μm-particle-size column was used. Data were ascertained by using the TOF instrument control software Mass Hunter Workstation A.02.00 and the Analyst software for data analysis.

Nuclear magnetic resonance analysis (NMR)

PHA samples were analyzed by NMR using a Bruker Avance AMX 400 instrument at 9.4 T. ^{1}H NMR chemical shifts in parts per million (ppm) were reported downfield from 0.00 ppm. A suspension containing 80 mg sample/2.0 mL chloroform-d ($CDCl_3$) was vortexed for 30 min, filtered (0.22-μm filter), and 1.0 mL placed into 5-mm NMR tubes. The following parameters were selected for analysis of samples: temperature, 30°C; sweep width, 20.69 ppm; 64 K data points; 30° excitation pulse; acquisition delay, 6 s. ^{13}C NMR data were ascertained with similar parameters and new samples. Acquisition time was set for 2 h/sample. Three independent runs were done for each method.

Gel permeation chromatography (GPC)

Gel permeation chromatography was used to ascertain the weight number average mass (M_n), molecular mass (M_w), peak molecular mass (MP), and polydispersity (M_w/M_n) of the PHA. Samples (1.0 mg) were dissolved in 50:50 tetrahydrofuran (THF; Fisher, HPLC grade)/chloroform (Fisher, HPLC grade) and filtered (0.2 μm). A Waters™ 2690 GPC separation module equipped with a Waters 2,410 refractive index detector and Polymer Laboratories C-Linear mixed-bed size exclusion columns (2 × 300 mm/7.5 mm) was used. The degasser was set at continuous with a pressure of 0.5 psi, and the lines at 100°C. Polystyrene standards A and B (Polyscience Corp. Warrington, PA, USA) with low polydispersity were used to generate a chromatogram and calibration curve. THF was used as the eluant at a flow rate of 0.7 mL/min at 35°C. Runs were done in triplicate with fresh samples.

Results

Batch fermentation under varying temperatures and pH

The effect of temperature at 25, 30, and 37 °C, and initial pH of 5, 6, 7, 8 and 9 on PHA yield was investigated. PHA yield (% wt/wt dcm) was 26.06, 5.7, and 3.45 at 25, 30, and 37°C, respectively at initial pH of 7.0. Similarly, yields were 0, 1.67, 26.06, 6.18, and 9.77 for pH 5, 6, 7, 8, and 9, respectively, at 25°C. Optimum PHA yield occurred at

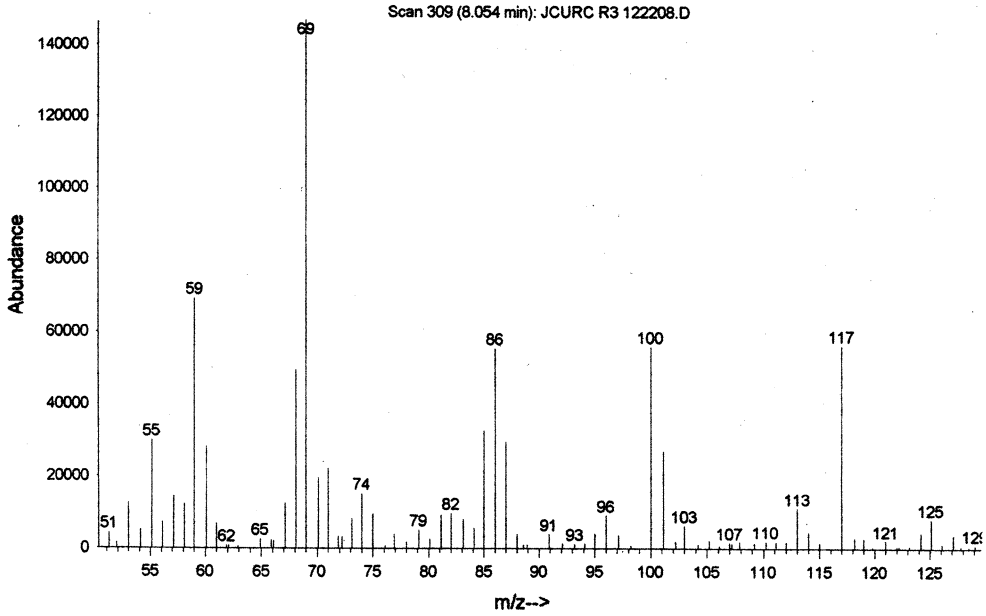

Fig. 1 Mass spectrum of transesterified polymer produced by *P. oleovorans* cultivated on saponified *Jatropha curcas* oil. The HB monomeric unit is indicated by the ion at *m/z* 117. The ions at *m/z* 100 and 86 are fragments from the ion at *m/z* 117. These indicate the presence of a butyrate unit in the original polymer

25°C and pH 7. Percentage yield was based on oleic acid. The melting point of the polymer was between 150 and 160°C.

Gas chromatography/mass spectrometry (GC/MS)

The mass spectrum for the *P. oleovorans* PHA illustrates the various fragments of the methyl-*β*-hydroxybutanoate monomers produced from base hydrolysis of polymers (Fig. 1). The $M(H)^+$ ion is identified at *m/z* 117 with characteristic fragments at *m/z* 100 and 85. The ions identified at *m/z* 117(+1) [$CH_3CH(OH)CH_2COOCH_3$], *m/z* 100 [$CH_3(CH)_2COOCH_3$], and *m/z* 85 [$CH_3CH(OH)CH_2CO$–]. Similar fragmentation patterns were seen for the commercial homopolymer poly(3-hydroxybutyrate), P(3HB) (data not shown).

Electrospray ionization–time of flight mass spectrometry (ESI–TOF MS)

ESI–TOF MS was used to determine the composition of base transesterified PHA produced by *P. oleovorans* (Fig. 2). The spectrum extends from *m/z* 1,145 to 1,200 and shows mostly sodium $[M + Na]^+$ and potassium $[M + K]^+$ adducts attached to the oligomeric chains of the PHA. The spectrum consists of clusters of isotopically resolved peaks of the same oligomer, but containing different end groups. Interpretation of the observed peaks is shown in Table 1. The ion at *m/z* 1,155.49 was identified as a sodiated dodecamer with an olefinic end group. The potassium adduct of this oligomer is shown at *m/z* 1,173.47. Likewise other notable peaks are shown at *m/z* 1,169, 1,184.47, and 1,194.01 which are attributable to oligomers containing both sodium and potassium adducts.

^{1}H and ^{13}C NMR analysis

^{1}H NMR spectroscopy was used to identify the protons of the PHA produced (Fig. 3). Peaks were identified as follows: the doublet at $\delta = 1.29$ ppm is attributable to the methyl group coupled to one proton; the double quadruplet at $\delta = 2.57$ ppm, a methylene group adjacent to an asymmetric carbon atom bearing a single proton; the multiplet at $\delta = 5.27$ ppm, a methine group; $\delta = 3.49$ ppm, methanol; the two broad signals at $\delta = 1.56$ and 7.25 ppm, water and chloroform respectively. The peaks at $\delta = 5.2$ and 5.185 ppm indicate (–CH_2) and (–C_2H_5) groups respectively. Peaks identified by ^{13}C NMR are referenced to $CDCl_3$ at 77.00 ppm; these were as follows: 169.9 ppm, a carbonyl carbon; 67.92 ppm, a methine carbon beta to a carbonyl carbon; 41.10 ppm, a carbon alpha to a carbonyl carbon; 30.01 ppm, an ethyl carbon; 20.08 ppm, a methyl carbon (Fig. 4).

Gel permeation chromatography (GPC)

GPC was used to determine the weight number average mass (M_n), peak molecular weight (MP), molecular weight (M_w), and polydispersity (M_w/M_n) of the polymer produced from *J. curcas* oil. Polystyrene standards A and B were used for the generation of a standard curve (data not shown). The peak molecular weight (MP) ranged from 1,300 to 377,400 Da for standard A and 580 to 210,500 Da for standard B. *J. curcas* PHA had an elution time of 14–20 min with an MP of 179,797 (data not shown).

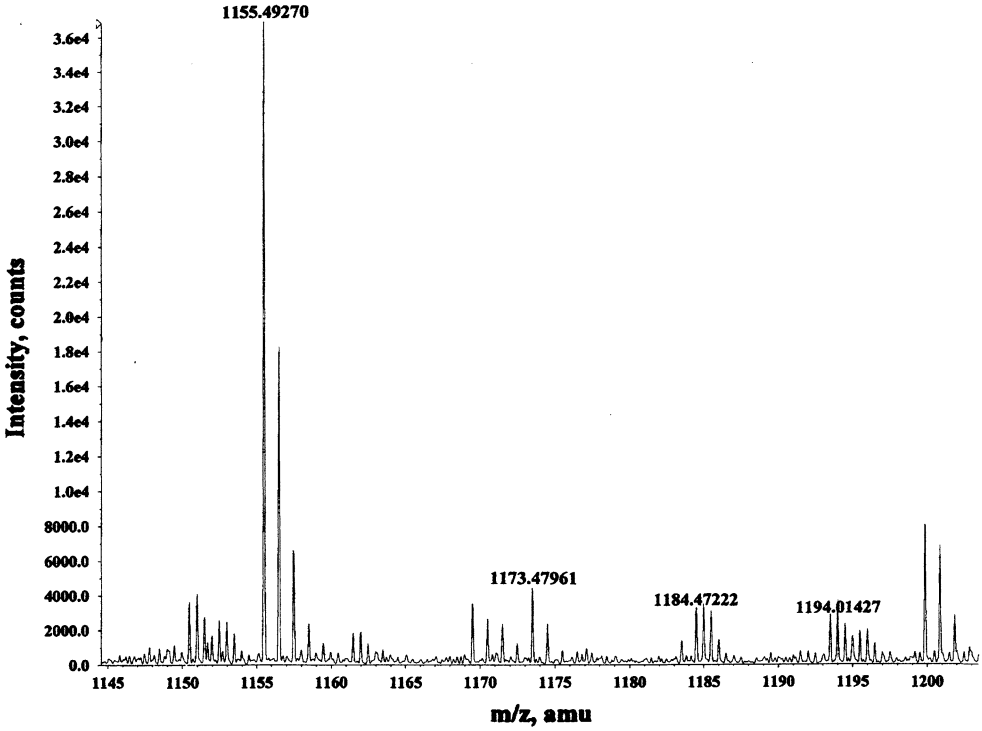

Fig. 2 Positive ion ESI–TOF MS of partially transesterified PHA oligomer produced by *Pseudomonas oleovorans* grown on saponified *Jatropha curcas* oil. Peak at *m/z* 1155.45 indicates the sodiated HB oligomer. The potassium adduct of this oligomer is shown at *m/z* 1,173.4. The peaks at *m/z* 1,169, 1,184.47, and 1,194.01 indicate the presence of the hydroxyvalerate (HV) units (see Table 1)

Table 1 Peak assignment of positive ion ESI–TOF mass spectrometry data for PHA oligomers identified in Fig. 2

Peak/ion	Measured mass	Calculated mass	Proposed oligomer
$[M + Na]^+$	1,155.49	1,155.53	$[CH_3CH{=}CHCO(HB)_{12}OCH_3{—}Na]^+$
$[M + Na]^+$	1,173.48	1,173.49	$[CH_3CH(OH)CHCO(HB)_{12}OCH_3{—}Na]^+$
$[M + Na]^+$	1,169	1,169	$[CH_3CH{=}CHCO(HB)_{11}(HV)_1OCH_3{—}Na]^+$
$[M + K]^+$	1,184.05	1,184.09	$[CH_3CH{=}CHCO(HB)_{10}(HV)_2OCH_3{—}K]^+$
$[M + K]^+$	1,194.01	1,194.06	$[CH_3CH{=}CHCO(HB)_9(HV)_3OCH_3{—}K]^+$

Discussion

In this study, the bacterium *P. oleovorans* (ATCC 29347) was able to use *J. curcas* seed oil as the only carbon source to synthesize the copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate, P(3HB-*co*-3HV), under batch fermentation. The oil has great potential for large-scale production of the polymer as it is renewable, generally not used for human consumption, and can be cheaply produced in large scale.

The optimum polymer yield of 26.06% per dry cell weight shows improvement over other studies where plant oils such as euphorbia, linseed, and other plants yielded 20, 20, and 15 to 23.5% PHA, respectively [6, 10, 41] (Table 2). Saponification of *J. curcas* oil was necessary since *P.oleovorans* does not produce lipases to metabolize triacylglycerols (TAGs) [8, 42]. The quantity of *J. curcas* seed oil in the fermentation medium was predetermined, and is comparable to that used in a previous study involving saponified vernonia oil [2]. The melting point (T_m) for the *J. curcas* PHA ranged between 150 and 160°C, which is characteristic of a copolymer but differs from the average T_m of 174–179°C typical of PHA homopolymer with 3HB monomer [22, 31].

To further verify the composition of the material, GC/MS analysis was done. The data identified only the methyl 3-hydroxybutanoate monomeric unit (3HB). However, since the T_m was below the range for the homopolymer (3HB) and the fact that pseudomonads are known producers of medium chain length PHA, we reasoned that the presence of only the 3HB monomer was unlikely. Therefore, to confirm this observation, further analysis of the PHA was undertaken by using the more sensitive mass spectrometric method electrospray ionization–time of flight (ESI–TOF) mass spectrometry. Data generated from ESI–TOF MS indicated that the PHA was a copolymer consisting of hydroxybutyrate (3HB) and hydroxyvalerate (3HV) units. The 3HV unit was identified by the peak at *m/z* 1,169, which indicates 1 valerate unit, i.e., HB_{11} HV_1; *m/z* 1,184, 2 valerate units i.e., $HB_{10}HV_2$; and *m/z* 1,194, 3 valerate units, i.e., HB_9 HV_3. These were of particular importance, since they identified the PHA as a copolymer consisting of

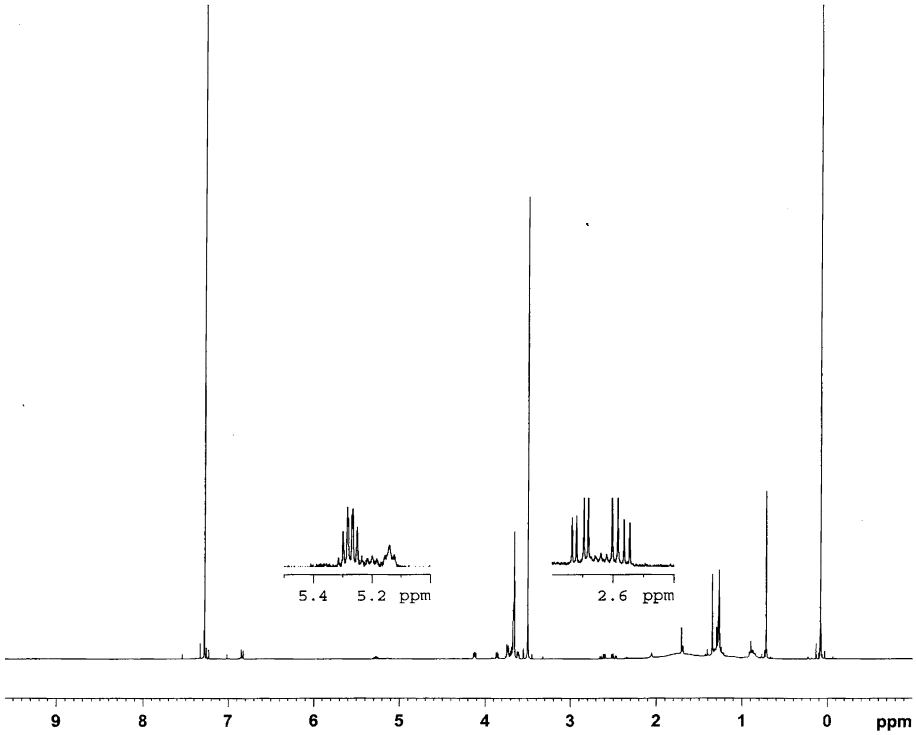

Fig. 3 ^{1}H NMR spectrum of PHA produced from saponified *J. curcas* oil by *P. oleovorans*. Characteristic PHA peaks were as follows: singlet at 0.1 ppm, TMS; doublet at 1.29 ppm, methyl group coupled to 1 proton; double quartet at 2.57 ppm, CH_2 adjacent to asymmetric carbon bearing single proton; multiplet at 5.27 ppm, methine; 7.25 ppm, chloroform

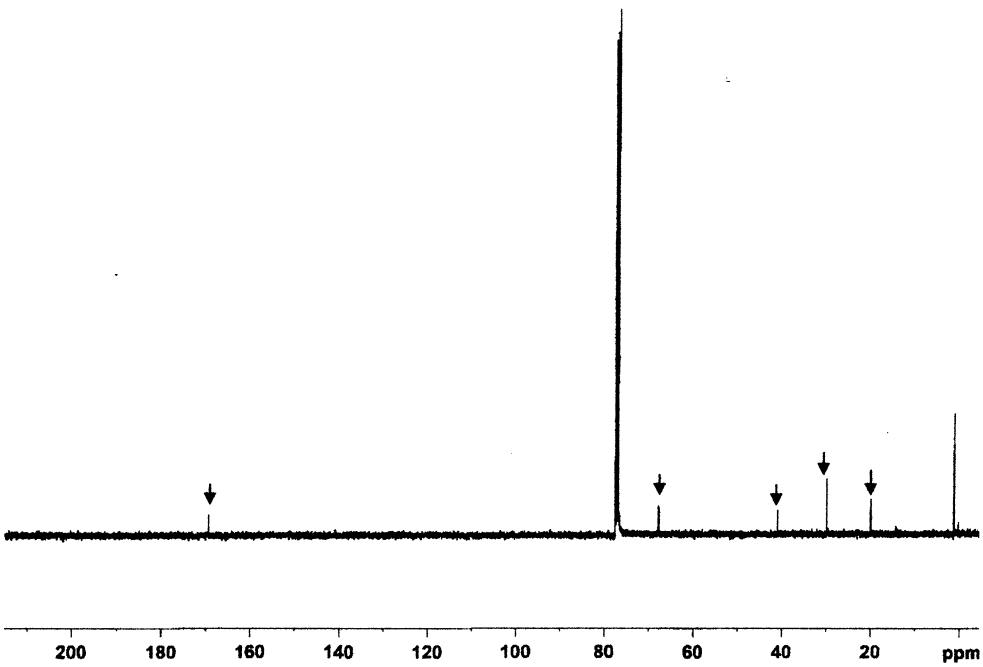

Fig. 4 ^{13}C nuclear magnetic resonance spectrum of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) produced from saponified *Jatropha curcas* oil by *Pseudomonas oleovorans*. Peaks are referenced to $CDCl_3$ at 77.00 ppm. Samples were 40 mg/mL in $CDCl_3$. Chemical shift (ppm): 169.1, carbonyl carbon; 77.0, 77.32, 77.63, $CDCl_3$; 67.92, methine carbon; 41.1, methylene carbon alpha to carbonyl carbon; 30.01, methylene carbon; 20.08, methyl carbon. *Arrows* indicate the location of carbon atoms

HB/HV and not a homopolymer. This was further confirmed by ^{1}H NMR spectroscopy. The hydroxybutyrate and hydroxyvalerate units were specifically identified by ^{1}H NMR by the peaks at $\delta = 5.2$ ppm and $\delta = 5.185$, respectively. The spectrum is consistent with previous findings [16, 39]. ^{13}C NMR identified the *J. curcas* PHA as a mixed polymer and supports the data from ESI–TOF MS, which indicate the presence of the valerate unit. The peak at 1.32 ppm was not identified but was also present in the ^{13}C NMR spectrum of the commercial homopolymer P(3HB).

Further characterization of the polymer by GPC indicated that it had polydispersity (M_w/M_n) of 1.3 and peak molecular weight (MP) of 1.79×10^5 which is similar to the characteristic range of 5×10^4 to 6×10^4 Da for the pseudomonads [30], and for other wild-type bacteria 1×10^4 to 3×10^6 with polydispersity of 2 [29]. The

Table 2 Summary of PHA production by the pseudomonads from various substrates

Strain	Substrate	Fermentation mode	Polymer	PHA content (%)	Reference
P. aeruginosa	Rice, canola, sunflower, corn, soybean oils	Batch	mcl-PHA[a]	15–23.5	[41]
P. aeruginosa	Euphorbia oil	–	PHA	20	[10]
P. oleovorans	Octanoic acid	Fed-batch	mcl-PHA	62–67	[23]
P. oleovorans	Octanoate	Chemostat	P[(*R*)-3-HA)[b]	–	[9]
Pseudomonas sp. El-2	Glucose	Fed-batch	P(3HB/3HV)[c]	53	[43]
P. putida	Oleic acid	Fed-batch	mcl-PHA	51.4	[26]
P. putida PGA1	Oleic acid	Fed-batch	mcl-PHA	44.8	[32]
P. putida KT2440	Nonanoic acid and glucose	Fed-batch	mcl-PHA	56	[45]
P. putida IPT046	Rice, canola, sunflower, corn, soybean oils	Batch	mcl-PHA	5.7–34.4	[41]
P. putida PGA1	Saponified palm kernel oil	–	PHA	37	[46]
P. putida	Hydrolyzed linseed oil	–	PHA	20	[6]
P. stutzeri	Soybean	Batch	3,6-Epoxy-7-nonene	63	[17]
P. oleovorans	*Jatropha curcas* oil	Batch	P(3HB/3HV)	26.06	This study

[a] Medium chain length polyhydroxyalkanoate

[b] Poly(3-hydroxyalkanoate)

[c] Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)

– not indicated

observed polydispersity suggests that the polymer has uniform chain length, i.e., only one length of polymer is present in the PHA.

It was of interest to find that *P. oleovorans* (ATCC 29347) produced short chain length (scl) PHA, instead of the typical medium chain length PHA [11, 14, 25, 36, 51], when grown on saponified *J. curcas* seed oil as the only carbon source. Scl-pHA production may have been influenced by culture conditions on PHA synthase or other inherent property/properties of the *J. curcas* oil. PHA synthase has been shown to incorporate significant amounts of scl hydroxyacids when the genes were expressed in PHA-negative mutants of *Pseudomonas* sp. 61–3 [34]. The monomeric units—hydroxybutyrate and valerate—of *J. curcas* PHA were confirmed independently by GC/MS, ESI–TOF MS, and ^{1}H and ^{13}C NMR spectroscopy. Further studies are underway to optimize the parameters required for copolymer production and to account for the current observation that scl-PHA was produced by *P. oleovorans*. This study constitutes the first report involving the production of the PHA copolymer P(3HB/3HV) from *J. curcas* seed oil by *P. oleovorans.*

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