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Synthesis and Physical Properties of Polyhydroxyalkanoate Polymers with Different Monomer Compositions by Recombinant *Pseudomonas putida* LS46 Expressing a Novel PHA SYNTHASE ($PhaC1_{16}$) Enzyme

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Abstract: A recombinant of *Pseudomonas putida* LS461 (deletion of the *phaC1phaZphaC2* genes) was constructed by introducing cosmid JC123 carrying a novel $phaC1_{16}$ gene from a metagenomic clone. The resulting strain, *P. putida* LS46123, was able to synthesize polyhydroxyalkanoate (PHA) polymers with novel monomer compositions when cultured on glucose or free fatty acids, and accumulated PHAs from 9.24% to 27.09% of cell dry weight. The PHAs synthesized by *P. putida* LS46123 contained up to 50 mol % short chain length subunits (3-hydroxybutyrate and 3-hydroxyvalerate), with the remaining monomers consisting of various medium chain length subunits. The $PhaC1_{16}$ protein expressed by *P. putida* LS46123 had an amino acid sequence similarity of 45% with the PhaC1 protein of the parent strain, *P. putida* LS46. Predicted 3D structures of the $PhaC1_{16}$ proteins from *P. putida* LS46123 and *P. putida* LS46 revealed several differences in the numbers and locations of protein secondary structures. The physical and thermal properties of the novel polymers synthesized by *P. putida* LS46123 cultured with glucose or free fatty acids differed significantly from those produced by *P. putida* LS46 grown on the same substrates. PHA polymers with different subunit compositions, and hence different physical and thermal properties, can be tailor-made using novel PHA synthase for specific applications.

Keywords: *Pseudomonas putida*; polyhydroxyalkanoates; PHA synthase; copolymers; physical properties of PHA polymers

1. Introduction

Polyhydroxyalkanoates (PHAs) have attracted extensive interest as environmentally friendly biodegradable plastics [1]. Many bacteria synthesize PHA polymers as carbon and energy storage reserves when they are grown under nutrient-limitation conditions [2]. PHAs have been classified as short chain length PHAs (SCL-PHA) and medium chain length PHAs (MCL-PHAs), based on the monomer composition of the polymers. The SCL-PHAs consist of 3-hydroxy fatty acid subunits containing four to five carbons, while MCL-PHA have 3-hydroxy fatty acid subunits containing six to 16 carbons. More than 100 different types of monomer units have been identified in PHA polymers [3].

PHA polymers are thermoplastic and differences in their physical and thermal properties are a function of their subunit composition. PHA polymers may be homopolymers containing only one type of 3-hydroxy fatty acid monomer, they may be copolymers containing two types of 3-hydroxy fatty acid monomers, or they may be heteropolymers containing 3-hydroxy fatty acids of several different chain lengths. Homopolymers, random copolymers, and block copolymers of PHA may be produced depending on the bacterial species and growth conditions [4].

Poly-(3-hydroxybutyrate), PHB, is a homopolymer containing four carbon subunits of 3-hydroxybutyrate (3HB), and the most widespread and best characterized member of the polyhydroxyalkanoate family [5,6]. PHB is highly crystalline (>50%), and consequently is relatively brittle and stiff, with a low elongation to break ratio. For these reasons, efforts in compounding PHB are mainly focused on the search of plasticizers and nucleating agents capable of reducing the crystallization process and improving flexibility. Copolymers containing a mixture of four and five carbon chain length subunits may be produced by culturing bacteria that synthesize these polymers with valeric acid, which results in the formation of PHAs containing 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) monomers. The incorporation of HV into PHB polymers results in a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB/3HV)], which is less stiff and brittle than pure P(3HB) [7].

Medium chain length PHAs are flexible and elastic, having low crystallinity with low tensile strength and high elongation-to-break ratios. In comparison to SCL-PHA, MCL-PHA polymers have low melting temperatures, low glass transition temperatures, and higher elongation-to-break ratios [8]. Copolymers of PHB and PHV with different mol % of 3HV (up to 71 mol % 3HV) were produced by *Alcaligenes eutropha* using different substrates [9,10]. Nodax™ class PHA copolymers produced by Meridian (Bainbridge, GA, USA) are also copolymers of 3HB and 3-hydroxyhexanoate (3HHx) fatty acid acids in varying proportions [11]. Recombinants of *P. putida* GPp104 carrying PHA synthase of *Aeromonas hydrophila* could add only 14 mol % 3HHx into copolymer [12]. The combination of 3-hydroxyhexanoate (3HHx) and 3-hydroxyocatanoate (3HO) subunits with 3HB subunits imparts significant changes in the thermal and physical properties of these copolymers. *Pseudomonas putida* LS46 synthesizes MCL-PHAs consisting of 3HHx, 3HO, 3HD), and/or 3-hydroxydodecanoate (3HDD) monomers in different proportions [10]. The *P. putida* LS46 genome was sequenced and regulatory genes and proteins associated with PHA synthesis were identified using transcriptomics and proteomic analyses [13–16]. The Type II PHA synthase enzyme (PhaC1, encoded by *phaC1*) expressed by *P. putida* is unable to add 3-hydroxybutyrate (3HB) monomers to MCL-PHA polymers.

Substrate specificity of PHA synthase is responsible for monomer composition. Few PHA synthases with wide substrate specificity have been identified in *Aeromonas caviae* and *Pseudomonas stutzeri* and recombinant expressing these PHA synthases in PHA negative mutant of *Ralstonia eutropha*, produced copolymer of SCL- and MCL-PHA copolymer [17]. Combinatorial mutations in *P. putida*, *P. oleovorans*, and *P. aeruginosa* with the ability to synthesize PHAs with 3HB, 3HHx, 3HO, 3HD, or 3HDD monomers have been described [18]. Two natural isolates of *Pseudomonas* sp. 61-3 and *Pseudomonas* sp. MBEL 6-19 were able to produce copolymers of 3HB with HHx, HO, and HD [19–21]. *Aeromonas hydrophila* and *Aeromonas caviae* are two other bacteria that express PhaC1 enzymes that can add both 3HB and 3HHx to PHAs [22–24]. Even the codon optimization of PHA synthase (*pha*C2ps) of *P. stutzeri* enhanced the incorporation of 3HB in copolymers [25].

New *phaC* genes that encode PHA synthase enzymes, and that can produce PHAs with novel monomer compositions, have been isolated from different bacteria in various environments [26–28]. DNA extracted from a soil microbial community was used to construct metagenomic libraries, which were then screened for novel *phaC* genes [29]. Cloning of these *phaC* genes and expression in heterologous systems led to the identification of novel PHA synthase (PhaC1 and PhaC2) enzymes. One of these novel *phaC* genes (encoding $PhaC1_{16}$ from a metagenomic clone 16) was cloned into the broad host-range cosmid pRK7813 [30,31]. We constructed a recombinant strain of *P. putida* LS46, designated *P. putida* LS46123, that expressed by the $phaC_{16}$ gene encoding the novel $PhaC1_{16}$ enzyme.

P. putida LS46123 produced copolymers with up to 50 mol % 3HB and 50 mol % MCL-PHAs. Here we report on the physical and thermal properties of PHA polymers with novel subunit composition synthesized by *P. putida* LS46123 cultured on fatty acids and glucose, as well as on differences in the amino acid sequences and predicted 3D structures of the PhaC1 enzymes expressed by *P. putida* LS 46 and *P. putida* LS 46123.

2. Materials and Methods

2.1. Strains and Plasmids

The strains, cosmid, plasmids, and primers used in present study are listed in Table 1.

Table 1. Bacterial strains, plasmids, cosmids, and primers used in this study. Restriction sites are underlined.

Strain/Plasmid/Primer	Characteristics	References
P. putida LS46	*P. putida* isolated from waste water	[13]
P. putida LS461	*phaC1-phaZ-phaC2* deletion mutant of *P. putida* LS46	This study
P. putida LS46123	*P. putida* LS461 carrying plasmid pJC123; Tc^R	This study
E. coli DH5α	Δ*lacZ*, ΔM15, Δ(*lacZYA-argF*), U169, *recA1*, *endA1*, *hsdR17*(rK-mK+), *supE44*, *thi-1*, *gyrA96*, *relA1*	Qiagen, Hilden, Germany
pRK7813	IncP oriT cos lacZα, Tc^R	[30]
pJC123	Derivative of pRK7813 carrying $phaC1_{16}$ gene from clone 16; Tc^R	[31]
pK18mobsacB	Narrow host-range cloning vector; *sacB*, Km^R	[32]
pRK2013	Helper plasmid pRK290 derivative; Km^R	[33]
pPHAC1C2	pK18*mobsac*B carrying 840 bp from *phaC1* and 857 bp from *phaC2* for operon knockout	This study
Pphac1forXbal	5′-CTAGTCTAGATGCGGTCAAACGCTTCTTCGAAAC-3′	This study
Pphac1revHindIII	5′-GATCAAGCTTGTCAGCGGGTTGCTCTTGAACATT-3′	This study
Pphac2forHindIII	5′-GATCAAGCTTAGACACGCCAGTGGATCGATGAAA-3′	This study
Pphac2revNhel	5′-CTAGGCTAGCTCTTGCCGAGCAGGTAGTTGTTGA-3′	This study

2.2. Construction of P. putida LS461 with Partial Deletion of the PHA Synthesis Gene Cluster

The PHA synthesis gene operon of *P. putida* LS46 was identified from the complete genome sequence (http//www.ncbi.nlm.nih.gov/nucore/ALPV02000008) coordinate 202310-197359; 13) and encoded the following genes: *phaC1phaZphaC2phaD*. A deletion mutant, designated *P. putida* LS461, was constructed by deleting the 3′-*phaC1*, *phaZ* and 5′-*phaC2* genes from the *P. putida* LS46 *pha* operon.

Primers were designed for the left and right flanking regions of the *phaC1phaZphaC2* genes (PPUTLS46_005625, 1683 bp; PPUTLS46_005620, 852 bp; and PPUTLS46_005611, 1680 bp) (Table 1). The size of nucleotide sequence spanning from *phaC1* to *phaC2* was 4325 bp. A partial sequence of *phaC1* (840 bp, from 426 to 1265 nt) was amplified with Primer 1 (Pphac1forXbal), which contained an XbaI restriction endonuclease site, and Primer 2 (Pphac1revHindIII), which contained a HindIII restriction endonuclease site. The amplified fragment was digested with restriction enzymes Xbal and HindIII and ligated into XbaI-HindIII digested pK18mobsacB vector [32], resulting in plasmid pPHAC1. Another fragment of 857 bp (from 2950 to 3807 nt) of the *phaC2* gene was amplified with Primer 3 (Pphac2forHindIII), which contained a HindIII site, and Primer 4 (Pphac2revNhel), which contained an NheI site. The amplified fragment was digested with HindIII and Nhel, and ligated into HindIII-NheI digested pPHAC1 to form pPHAC1C2. Plasmid pPHAC1C2 had a deletion of the 3′-*phaC1* gene, complete *phaZ* gene, and 5′-*phaC2* gene.

The plasmid pPHAC1C2 was a narrow host range plasmid with deletion in the *phaC1phaZphaC2* genes. This plasmid was transferred to *P. putida* LS46 by triparental mating and transconjugants were selected on LB plates containing chloramphenicol (200 μg/mL) and kanamycin (50 μg/mL) [31]. Two kanamycin-resistant clones were selected and grown in 10 mL LB broth at 30 °C for 18 h.

Serial dilutions of the culture were spread on LB plates with 5% (*w*/*v*) sucrose. Clones were screened for the loss of the kanamycin marker. One kanamycin-sensitive clone obtained in this manner (*P. putida* LS461) with a deletion of the *phaC1phaZphaC2* region was confirmed by PCR amplification, The PHA synthesis phenotypes of the parental strain *P. putida* LS46 and the deletion mutant *P. putida* LS461 were compared using Ramsay Minimal Media (RMM) containing glucose as the sole carbon source.

2.3. Construction and Transfer of pJC123 into P. putida LS461

The $phaC1_{16}$ gene of metagenomic clone 16 was amplified by PCR and cloned as a HindII-EcoRI fragment into cosmid pRK7813 to give plasmid pJC123. The $phaC1_{16}$ gene was expressed from constitutive *lacZ* promoter [31]. *Pseudomonas putida* LS461 with deletion in *phaC1phaZphaC2* was used to express a heterologous $phaC1_{16}$ gene. Cosmid pJC123 was transferred into *P. putida* LS461 by triparental mating by using pRK2013 as a helper plasmid [33,34]. Transconjugants were selected on chloramphenicol (200 µg/mL) and tetracycline (40 µg/mL). Clones were streak purified on the same medium and tested for PHA production. The resulting *P. putida* LS461 transconjugant was named *P. putida* LS46123.

2.4. PHA Synthesis

A single colony of *P. putida* LS46123 was inoculated in 100 mL LB broth with 40 µg/mL tetracycline. The inoculum culture was grown at 30 °C for 18 h on a rotary shaker (150 rpm). Ramsay's Minimal Medium (RMM, 100 mL) [8] plus different carbon sources and 40 µg/mL tetracycline was inoculated with 2% *v*/*v* inoculum culture. The different carbon sources used were glucose, hexanoic, octanoic, nonanoic, and decanoic acids, as well as biodiesel-derived free fatty acids. Glucose and the medium chain length fatty acids were obtained from Sigma Aldrich (St. Louis, MO, USA). Crude biodiesel-derived (waste) free fatty acids extracted from crude biodiesel-derived glycerol (REG-FFA) were obtained from the Renewable Energy Group LLC (Danville, IL, USA). The test cultures were incubated at 30 °C on a rotary shaker (150 rpm). At 72 h post-inoculation (hpi), the cultures were harvested by centrifugation at 4190× *g* for 10 min. All tests were conducted with three independently replicated cultures (i.e., three biological replicates). Cell biomass, PHA production (% cell dry weight, cdw), and PHA monomer composition were determined as described earlier [13].

2.5. PHA Production by P. putida LS46123 in Medium with Different Carbon Sources

P. putida LS46123 was grown in 50 mL LB broth with 30 µg/mL tetracycline for 18 h at 30 °C on a rotary shaker at 150 rpm (OD_{600} = 1.00). One hundred milliliters of RMM in 500-mL baffled flasks with different carbon sources and tetracycline (30 µg/mL) was inoculated with 1% inoculum (*v*/*v*) of *P. putida* LS46123 (OD_{600} = 1.0). Cultures were incubated at 30 °C on a rotary shaker. At 72 hpi, cells were centrifuged at 4190× *g* for 20 min. The cell pellet was washed twice in 0.9% NaCl and dried at 60 °C for 48 h to measure cell dry mass. PHA composition was determined by gas chromatography–mass spectrometry (GC-MS) analysis, as described [13].

2.6. Production and Purification of PHAs

Large-scale production of PHAs from *P. putida* LS46123 was carried out in batch cultures in a 13 L Applicon bioreactor. The bioreactor was autoclaved with 10 L RMM with different carbon sources. Tetracycline (30 µg/mL) was added when the culture broth had cooled to room temperature. Bioreactor conditions were set at 30 °C, pH 7.0, with stirring at 300 rpm, and dissolved oxygen at 30%. Culture was harvested after 48 h and cell biomass was recovered by centrifugation at 4190× *g* for 20 min. The pellet was washed twice in 0.9% NaCl and dried at 80 °C for 48 h to estimate cell dry mass. PHAs were extracted from the dry cell mass with chloroform in Soxhlet apparatus for 6 h. PHAs were concentrated by evaporating the chloroform and then precipitated using cold methanol. Supernatant was decanted and methanol was then evaporated. The precipitation and evaporation steps were repeated again and purified PHAs were stored for further analysis.

2.7. Physical Properties of Polymer Produced by P. putida *LS46123*

2.7.1. NMR and FTIR Spectra of PHA Polymers

^{1}H-NMR spectra of PHA polymers synthesized by *P. putida* LS46123 were acquired by dissolving 10 mg of each polymer in deuterochloroform ($CDCl_3$) and analyzed on a Brucker AMX-300 spectrometer (Brucker Biospin AG, Billerica, MA, USA) at 22 °C with 7.4 ms pulse width (30° pulse angle), 1 s pulse repetition, 10,330 Hz spectral width, and 65,536 data points and analyzed as described by Huijbert et al. [35] FT-IR analysis of each polymer sample was carried out on a MB-3000, ABB FTIR spectrophotometer (ABB, Quebec, QC, Canada) in range 600–4000 cm^{-1} and were analyzed as described by Hong et al. [36].

2.7.2. Viscosity of PHAs

Four PHA polymers produced by *P. putida* 46123 using hexanoate, octanoate, nonaote, and biodiesel fatty acids were studied for viscosity using a Brookfield DV-II Pro (Brookfield, Middelboro, MA, USA). Ten grams of PHAs were dissolved in chloroform to a volume of 20 mL. The sample (15 mL) was placed in conical centrifuge tube. A spindle (No. 68) was attached to a viscometer (make and model) and dipped in the polymer solution. The viscosity was estimated at different motor speeds as described by the Brookfield user manual.

2.7.3. Differential Scanning Calorimetric (DSC) Analysis

Differential scanning calorimetric (DSC, Columbus, OH, USA) analysis was performed using a Mettler Toledo DSC 3 instrument. The temperature was ramped at a heating rate of 10 °C/min under nitrogen to 200 °C.

2.7.4. Thermal Gravimetric Analysis (TGA)

TGA analysis was performed using a TGA instrument (Mettler-Toledo, TGA/SDTA 851, Columbus, OH, USA) calibrated with indium. The temperature was ramped at a heating rate of 10 °C/min under nitrogen to 200 °C.

2.7.5. Rheological Properties of PHA Polymers

Rheological properties of the PHA polymers were studied using a TA Discovery HR2 Rheometer (TA Instruments, New Castle, DE, USA). Properties like storage modulus, loss modulus, tan delta and complex viscosity were determined at different angular frequencies from 0.1 to 200.

2.8. Comparison of P. putida *LS46 and* P. putida *LS46123 PhaC1$_{16}$ Proteins*

The nucleotide and amino acid sequences of the *phaC1* genes and PhaC1 proteins, respectively, of *P. putida* LS46 and clone 16 were compared using NCBI protein BLASTP (www.ncbi.nih.nml.gov). For this *P. oleovorans* PhaC1 (WP_037049875.1), *P. putida* KT2440 (NP_747105.1), *P. putida* LS46 (EMR48251), *P. mendocina* ymp (ABP83300), and clone 16 (ALV86417.1). The 3D structures of the PhaC1 proteins were predicted using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2) [37].

3. Results

3.1. Cell Biomass and PHAs Production

Like *P. putida* LS46, *P. putida* LS46123 was able to utilize glucose, hexanoate, octanoate, nonanoate, decanoate, and biodiesel-derived free fatty acids (REG-FFA) as carbon sources for growth and PHA synthesis. However, the cell mass produced by *P. putida* LS46123 was very low compared to the cell mass produced by *P. putida* LS46 cultured with the same substrates (Table 2). The greatest cell mass production (g/L) was observed in RMM-glucose cultures, while the lowest cell mass production was

observed in RMM-hexanoate and RMM-octanoate cultures. In comparison to *P. putida* LS46, PHA accumulation by *P. putida* LS46123 was low in all the cultures with different substrates, and ranged from 9.3% to 27.1% of cell dry weight.

Table 2. Cell biomass and PHA yields by *Pseudomonas putida* LS46123 using different carbon substrates.

Strains	Carbon Source	Cell Biomass (g/L)	% PHA/cdw
P. putida LS46123	Glucose (2% *w*/*v*)	2.2 ± 0.3	9.2 ± 3.2
	Hexanoate (20 mM)	1.1 ± 0.2	16.2 ± 2.1
	Octanoate (20 mM)	1.1 ± 0.1	17.3 ± 1.5
	Nonanoate (20 mM)	1.4 ± 0.2	10.5 ± 0.8
	Decanoate (20 mM)	1.6 ± 0.1	14.1 ± 0.8
	REG-FFA (2% *v*/*v*)	2.1 ± 0.2	27.1 ± 1.7
P. putida LS46	Glucose (2% *w*/*v*)	2.7 ± 0.4	22.6 ± 0.8
	Hexanoate (20 mM)	2.1	19.1
	Octanoate (20 mM)	2.5	48.9
	Nonanoate (20 mM)	2.4	28.1
	Decanoate (20 mM)	2.5	33.7
	REG-FFA (2% *v*/*v*)	4.6	40.3

3.2. Monomer Composition of PHAs Produced by P. putida LS46123

P. putida LS46 can synthesize MCL-PHA polymers containing 3hydroxyhexanoate, 3hydroxyoctanoate, and 3hydroxydecanoate subunits as major components in cells grown with glucose, octanoate, decanoate, and REG-FFAs [10]. However, the monomer compositions of PHA polymers synthesized by *P. putida* LS46123 were very different from the compositions of polymers synthesized by the parent strain, *P. putida* LS46, on the same substrates. *P. putida* LS46123 added both short chain and medium chain length monomers to the PHA polymers it synthesized (Figure 1). In RMM-glucose cultures, *P. putida* LS46123 synthesized polymers consisting of 32% 3HB, 50% 3HHx, and 16% 3HO subunits, whereas in RMM containing hexanoic, octanoic and decanoic acid as substrate it accumulated 30%, 52%, and 45% of 3HB monomer. RMM-REG-FFA cultures the polymers synthesized by *P. putida* LS46123 consisted of up to 52% 3HB.

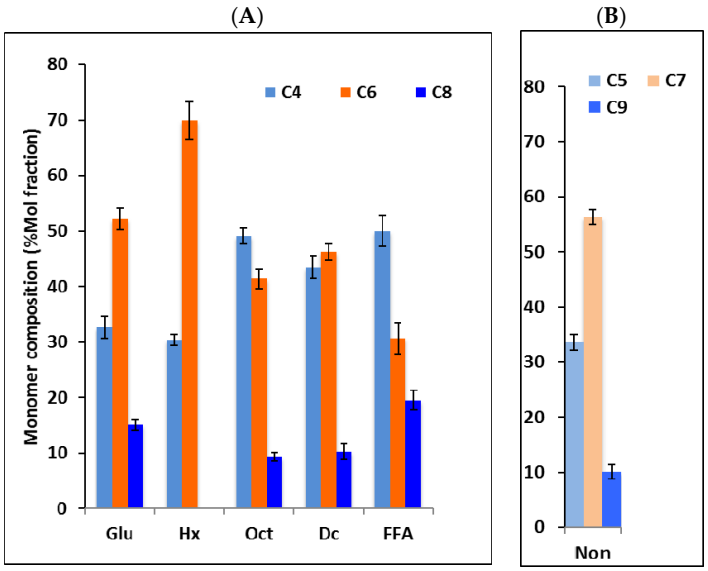

Figure 1. Monomer composition of PHA polymers synthesized by *P. putida* LS46123 grown on different carbon sources: (**A**) Glu, Glucose; Hx, Hexanoic acid; Oct, Octnoic acid; Dc, Decanoic acid; FFA, Biodiesel-derived free fatty acids. Subunit components: C4, 3-hydroxybutanoic acid; C6, 3-hydroxyhexanoic acid; C8, 3-hydroxyoctanoic acid; (**B**) Non, Nonanoic acid. Subunit components: C5, 3-hydroxyvaleric acid; C7, 3-hydroxyheptonoic acid; C9, 3-hydroxynonanoic acid.

With nonanoic acid as the substrate, it produced a polymer with 34 mol % 3HV, 56 mol % 3HHp, and 10 mol % 3HN. These monomer compositions were not only different from the polymers produced by parent strain *P. putida* LS46 but also different from all other known polymers produced by *P. putida* or *Ralstonia eutropha* strains.

3.3. Physical Properties of Polymer Produced by P. putida *LS46123*

NMR and FITR Spectra

^{1}H NMR spectra identify the origin of protons from different functional groups, and protons in five functional groups in the PHA polymers were identified by specific peaks in the ^{1}H NMR spectra. An example ^{1}H NMR spectrum of PHA polymer synthesized by *P. putida* LS46123 grown on nonanoic acid is shown in Figure 2. The five peaks corresponded to protons located in: (i) the terminal methyl group (CH_3); (ii) a free methylene group (CH_2); (iii) a methylene group attached to a carbonyl group; (iv) a methylene group next to the hydroxyl group; and (v) the first methylene group of saturated side chains. The ^{1}H NMR spectra indicated wavelength shift of 2.5, 5.2, 1.6, 1.2, and 0.8 ppm, respectively. These are signature peaks of polyhydroxyalkanoates. The five peaks identified in PHA polymers synthesize by *P. putida* LS46123 have also been identified in NMR spectra of PHAs produced by *P. putida* KT2440 [35]. Other peaks indicating unsaturated carbon double-bonds (C=C) in PHA side chains were not identified in polymers synthesized by *P. putida* LS46123 grown on octanoic and nonanoic acids, but were present in the PHAs produced by *P. putida* LS46123 grown on biodiesel-derived free fatty acids. Integration of proton peak from terminal methyl group and GC analysis confirmed the presence of SCL-PHAs along with MCL-PHAs.

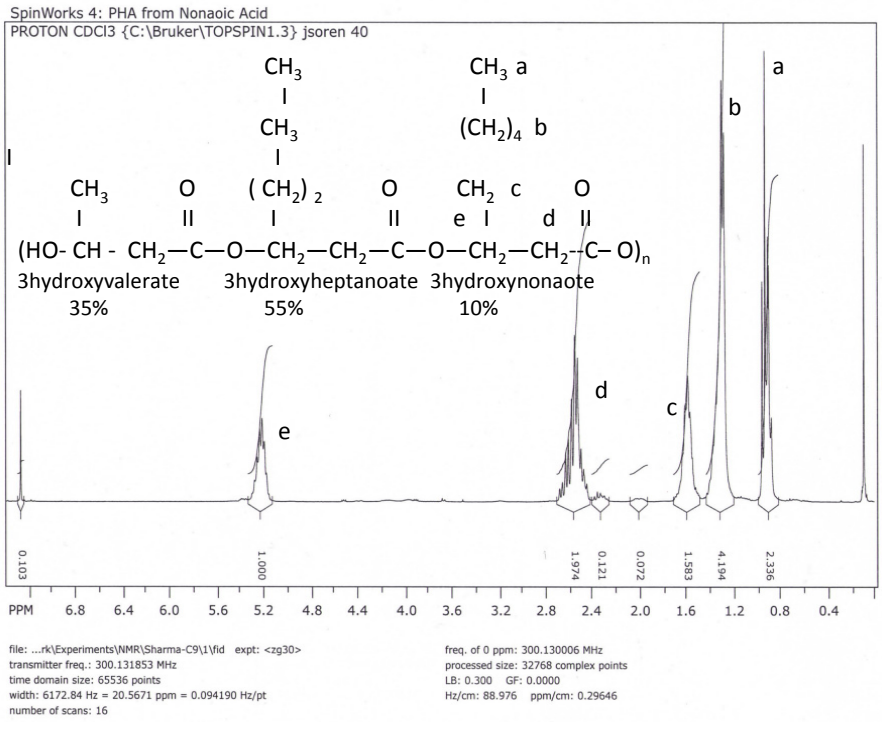

Figure 2. ^{1}H-NMR spectrum of PHA isolated from *P. putida* LS46123 grown in RMM medium with nonanoic acid.

The chemical structures of PHA polymers synthesized by *P. putida* LS46123 using different carbon substrate were evaluated using FT-IR spectroscopy. FT-IR analysis can identify the presence of different functional groups, such as aliphatic C–H bonds, =C–H bonds, =CH bonds, and =C–O bonds, as well as =O bond stretching, and =C–H bond deformation. It has been established that carbonyl group

peaks identified by FT-IR differ in PHB, MCL-PHAs, and PHB+PHA co-polymers. Hong et al. [36] determined that the carbonyl peaks in PHB polymers were at 1728 cm^{-1}, while the carbonyl peaks in MCL-PHA polymers were at 1740 cm^{-1}, and the carbonyl peaks in PHB + PHA co-polymers were at 1732 cm^{-1}. PHA polymers synthesized by *P. putida* LS46123 grown on hexanoic acid, octanoic acid, nonanoic acid, and REG-FFAs had carbonyl peaks at 1734, 1732, 1734, and 1734.7 cm^{-1}, respectively (Figure 3). The methylene C-H peaks (located at 2928 cm^{-1}) that are more prominent in MCL-PHAs than in PHBs were also detected in polymers synthesized by *P. putida* LS46123.

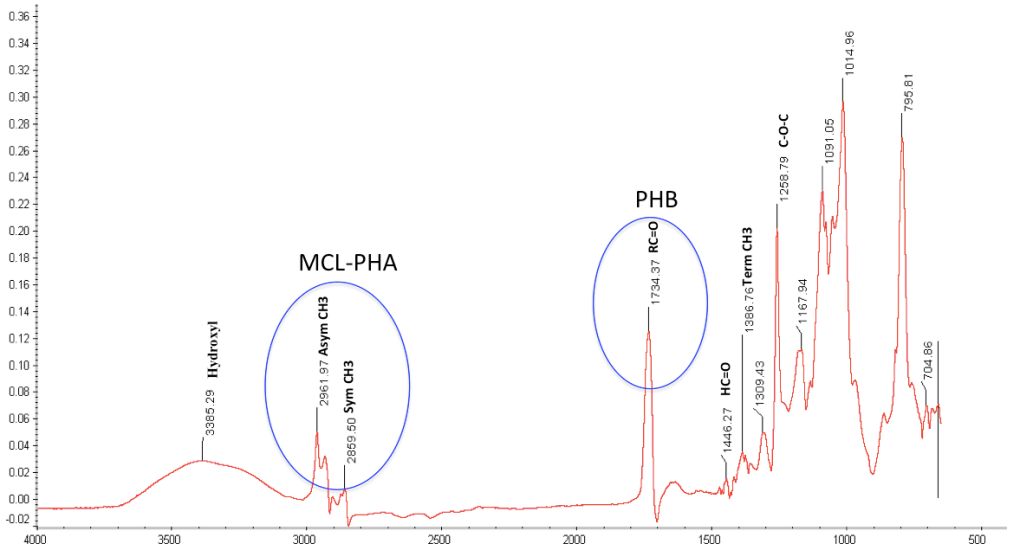

Figure 3. FT-IR spectrum of PHA polymer synthesized by *P. putida* 46123 grown on octanoic acid.

3.4. Glass Transition, Melting, and Thermal Degradation Temperatures

The glass transition temperatures (T_g) and melting temperatures (T_m) of PHA polymers synthesized by *P. putida* LS46123 were different from those of polymers produced by *P. putida* LS46. The T_g of different polymers synthesized by *P. putida* LS46123 were in the range of −28.7 to −34.7 °C (Table 3; Figure 4), and the T_m ranged between 138 °C and 166 °C. Polymer produced from decanoic acid had highest T_m (166 °C), while the polymer produced from hexanoic acid had the lowest T_m (138 °C). Thermal degradation temperatures (T_d) for different PHAs produced by *P. putida* LS46123 were in range of 248.6 °C to 258.3 °C, which were similar to PHAs produced by *P. putida* LS46 (Figure 5).

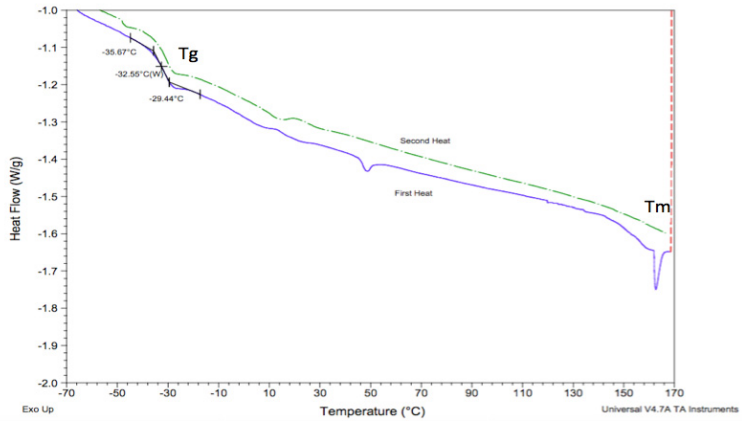

Figure 4. Differential Scanning Calorimetric (DSC) analysis of PHA polymer synthesized by *P. putida* 46123 grown on octanoic acid.

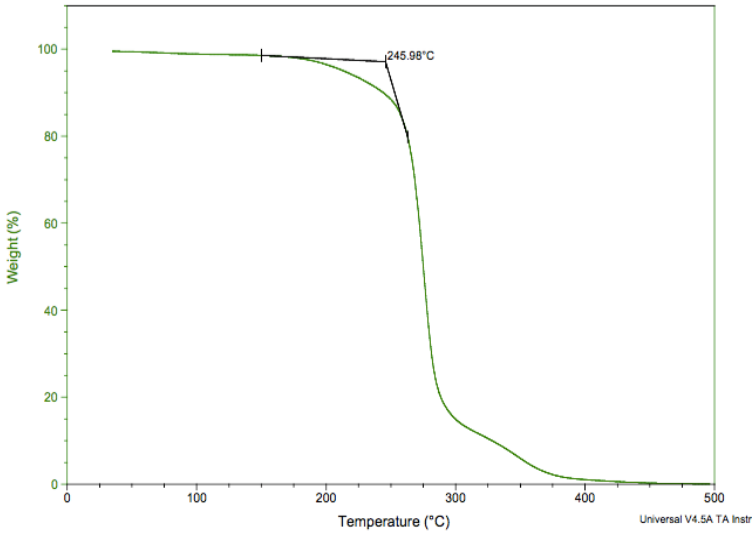

Figure 5. Thermal degradation (T_g) of PHA polymer synthesized by *P. putida* LS46123 grown on octanoic acid.

Table 3. Glass transition and melting temperatures of PHA polymers synthesized by *P. putida* LS46123 when grown on different fatty acids.

Substrate for PHA Production	Glass Transition Temperature (T_g °C)	Melting Temperature (T_m°C)	Degradation Temperature (T_d °C)	Wave Number (cm^{-1})	
				C=O	C–O
Hexanoic Acid	−28.7	138	254.5	1732	1170
Octanoic Acid	−32.6	162	258.3	1734	1167
Nonanoic Acid	−30.2	158	254.5	1734	1165
Decanoic Acid	−31.0	166	ND#	ND	ND
Biodiesel fatty acid	−34.4	140	248.6	1734	1177
Polyhydroxyalkanoates P(3HA)	−44.0	42	260	1742	1165
* Homopolymer of PHB	9.0	178	280	1728	1185
* Copolymer P(3HB-co-3HV)	−6.0 to −10.0	137–170	275	ND #	ND #

* Mozejko-Ciesielska and Kiewisz [38]; ND #, Not Determined.

3.5. Rheological Properties of PHA Synthesized by P. putida *LS46123*

PHAs produced by *P. putida* LS46123 grown on different substrates differed in their rheological properties. PHAs produced from hexanoic acid had highest viscosity, followed by PHAs produced from nonanoic acid. In contrast, polymers produced by *P. putida* LS46123 grown on octanoic acid and REG-FFAs had lower viscosity (Figure 6).

Rheological properties like storage modulus, loss modulus, tan delta, oscillation torque, and complex viscosity were estimated at different angular velocity of PHAs. PHAs synthesized by *P. putida* LS46123 grown on octanoic acid had a lower storage modulus (an indicator of elastic property) than polymers produced on hexanoic, nonanoic, and REG-FFAs. The Loss moduli (an indicator of viscosity) of PHAs produced on hexanoic and nonanoic acids were lower than PHAs produced on octanoic and REG-FFAs (Figure 7). Storage and loss modulus show elastic and viscous proportion. The Tan delta values, which are used to determine viscoelastic behavior of polymers (ratio of loss modulus to storage modulus), were lower for PHA polymers produced from hexanoic and nonanoic acids compared with polymers produced on octanoic and REG-FFAs (Figure 8).

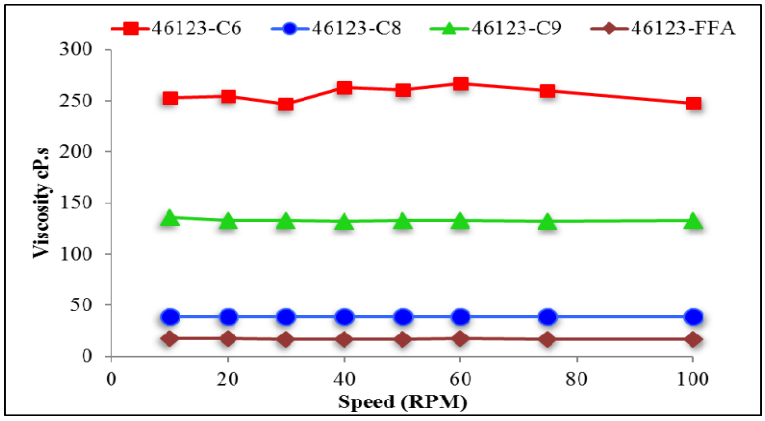

Figure 6. Viscosity of different polymers synthesized by *P. putida* LS46123 on different substrates. 46123C6, Hexanoic acid; 46123C8, Octanoic acid; 46123C9, Nonanoic acid; and 46123FFA, Biodiesel-derived free fatty acids (REG-FFAs).

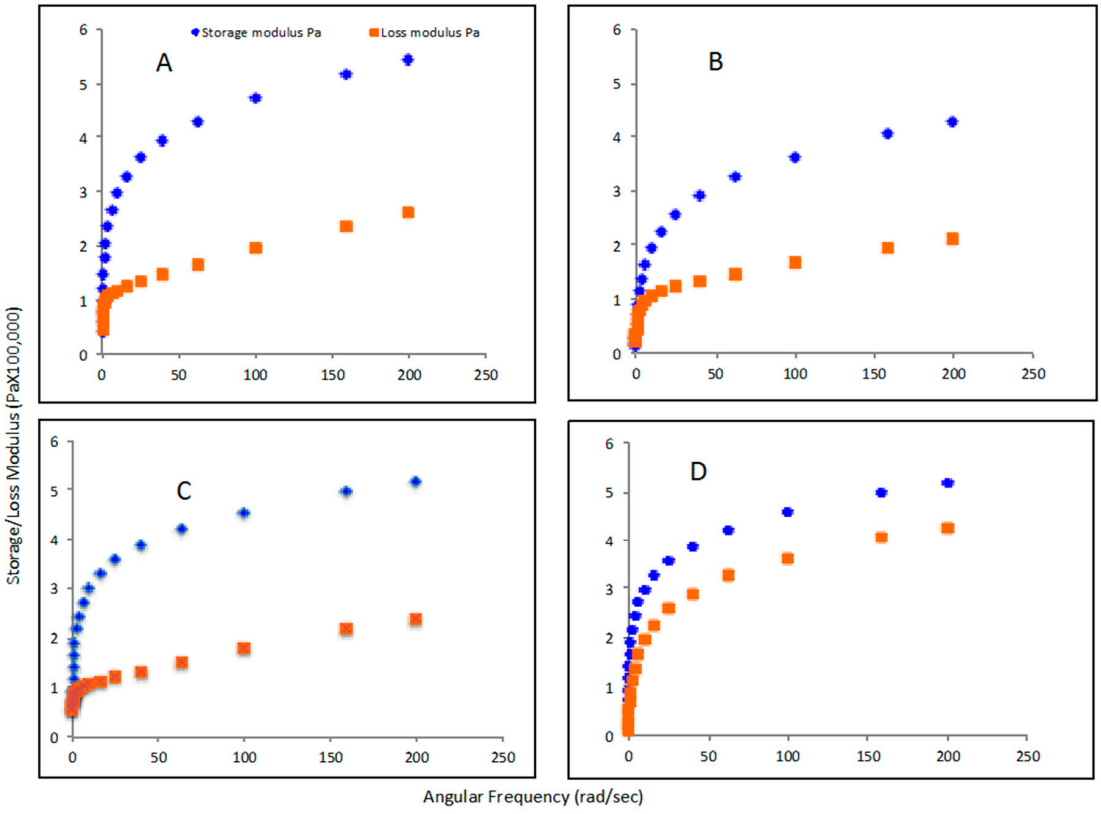

Figure 7. Storage modulus (G′) and loss modulus (G″) vs. angular frequency of PHA polymers synthesized by *P. putida* LS46123 grown on: (**A**) Hexanoic acid; (**B**) Octanoic acid; (**C**) Nonanoic acid; and (**D**) Biodiesel-derived free fatty acids (REG-FFAs).

Complex viscosity, which is a function of angular frequency, was higher for PHAs produced from hexanoic acid and nonanoic acid than polymers produced from octanoic acid and REG-FFAs (Figure 9). Overall, these data suggest that PHAs synthesized by *P. putida* LS46123 grown on hexanoic and nonanoic acids are more elastic compared with polymers produced by *P. putida* LS46123 grown on octanoic acid and biodiesel-derived free fatty acids.

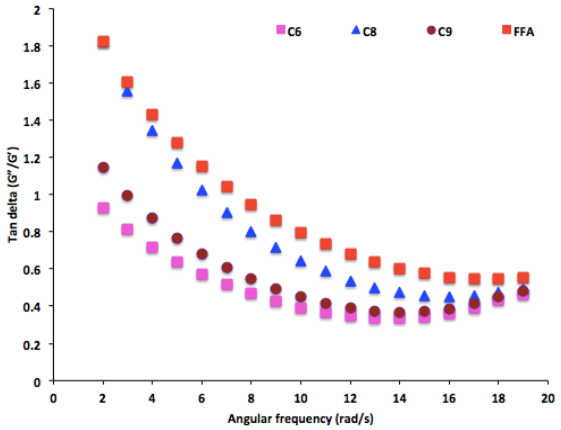

Figure 8. Tan delta (G″/G′ ratio) of PHA polymers synthesized by *P. putida* LS46123 grown on: C6, Hexanoic acid; C8, Octanoic acid; C9, Nonanoic acid; and FFA, biodiesel-derived free fatty acids (REG-FFA).

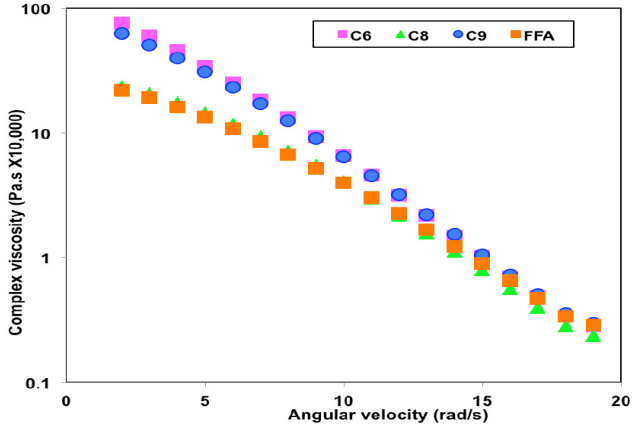

Figure 9. Complex viscosity of PHA polymers synthesized by *P. putida* LS46123 grown on: C6, Hexanoic acid; C8, Octanoic acid; C9, Nonanoic acid; and FFA, biodiesel-derived free fatty acids (REG-FFA).

3.6. Comparison between PhaC1 Proteins of P. putida LS46 and P. putida *LS46123*

The *phaC1*$_{16}$ gene was isolated from a metagenomic library prepared from soil and cloned [26] and subsequently expressed in *P. putida* LS46123. Analyses of the nucleotide and amino acidsequences of *phaC1*$_{16}$ revealed that it was closely related to the *phaC1* gene of *Pseudomonas* sp. F15. PhaC1$_{16}$ protein had a 48% amino acid sequence identity to PhaC1 protein of *Pseudomonas* sp. TTU2014-080ASC, and an 84% sequence identity to a PhaC1 protein from an uncultured clone 50 (sequence ID ALV86626). *P. putida* LS46 PhaC1 protein had an amino acid sequence identity of 46% with the PhaC1$_{16}$ protein (EMR48251). Other PhaC1 proteins from *P. mendocina*, *P. alcaligenes*, *P. stutzeri*, *P. aeruginosa*, and *Pseudomonas* sp. 61-3 had 45% to 47% similarity to the PhaC1$_{16}$ protein. These Pseudomonads are also known to produce SCL- and MCL-PHAs.

The amino acid sequences of the *P. putida* LS46 PhaC1$_{ls}$ protein were aligned with those of the *P. putida* KT2440 PhaC1$_{pp}$, *P. mendocina* ymp, *P. oleovavorans* PhaC1$_{po}$, and PhaC1$_{16}$ proteins (Figure 10). The amino acid position numbers in Table 4 are derived from the alignment of PhaC1$_{po}$ with wild-type and mutant PhaC1 proteins from *P. putida* Gpo1 that resulted in high incorporation of 3-hydroxybutyrate in co-polymers (Table 4) as described earlier [39], whereas the amino acid position numbers in Figure 10 are based on the alignment of the PhaC1 protein of *P. putida* LS46 with PhaC1$_{16}$ [39]. The amino acid sequences of the four PhaC1 proteins showed three gaps when aligned with the Clone 16 PhaC1$_{16}$. Consequently, the amino acid positions indicated in Table 4 and Figure 10 do not correspond.

Table 4. Comparison of amino acid sequences of PhaC1 protein from *P. putida* LS46 with those of the metagenomic library clone 16 ($PhaC1_{16}$) protein, and mutations in the PhaC1 of *P. putida* Gpo1 * that result in high incorporation of 3-hydroxybutyrate in co-polymers.

PhaC1 Enzyme	Mol % of 3HB	Amino Acid Substation in Conserved Regions of PhaC1 Proteins																				
		F1			F2						F3			F4				F5				F6
		228	230	231	292	293	295	297	377	379	399	401	403	404	481	482	483	484	520	521	523	524
Wild [1]	12	F	V	F	M	L	A	S	N	W	N	T	R	L	Q	S	I	L	L	H	Q	T
pL1-6	60		I	L	V		V	V	T	L					A	C		I	P		L	G
pD7-47	7		I	L	V		V	V	T	L					A	C		I	P		L	G
pPS-A2	36	Y	I	L	A	V	V	V		V					A	G		I	P		L	D
pPS-C2	50	Y	I	L	A	M	V	V		V				M	A	G		V	P		L	G
pPS-E1	45		I	L	T		V	V	T						A	C	V	V	P	D	L	E
LS46 [2]	–	F	V	F	M	L	A	S	N	W	N	T	R	L	Q	S	I	L	L	H	Q	S
$PhaC1_{16}$ [3]	Up to 50	Y	V	L	F	K	L	A	N	W	S	S	R	L	Q	T	L	V	E	M	I	K

* Modified from [40]; Numbering of amino acids in PhaC1 is based on Sheu and Lee [39]; [1] *P. putida* Gpo1 $PhaC1_{Pp\ Gpo1}$ (WP_037049875.1); [2] *P. putida* LS46 $PhaC1_{Pp\ LS46}$ (EMR48251), [3] Clone 16 $PhaC1_{16}$ (ALV86417.1); – indicates that no 3HB was produced.

Six conserved regions have been earlier identified in Class I and Class II PhaC1 proteins and mutation in these regions affect substrate specificity of PHA synthase. The amino acid sequence of conserved regions of $PhaC1_{ls}$ and $PhaC1_{16}$ showed some similarities and differences (Table 4). All PhaC1 have a Lipase Box sequence required for PhaC activity (Figure 10). In the first conserved region of the *P. putida* LS46 PhaC1 protein, two phenylalanine (F229 and F232) were replaced by tyrosine (Y229) and leucine (L232) in the $PhaC1_{16}$ (Table 4; Figure 10).

Differences between the $PhaC1_{16}$ and the $PhaC1_{po}$ proteins were also detected in the F2, F3, F4, F5, and F6 conserved regions of the protein (Table 4). Four amino acids in F2, two in F4, three in F5, and four in F6 were different in $PhaC1_{16}$ compared with $PhaC1_{ls}$. Some of the changes in amino acid corresponded to changes in the mutated $PhaC1_{po}$, which have been shown to be responsible for the addition of greater mol % of 3HB in polymers. Ser 325 is a critical amino acid in Class II PHA synthases that synthesize MCL-PHAs, and, with three other key amino acid residues—Glu130, Ser477, and Gln481—has been shown to be associated with specificity of the PHA synthase enzyme (PhaC1) in *P. putida* KT2440. However, no differences in in key amino acids associated with PHA Synthase catalytic site (Cys296, Asp451, and His479) were observed between the endogenous $PhaC_{ls}$ of *P. putida* LS46 and $PhaC1_{16}$ expressed in *P. putida* LS46123.

```
                10        20        30        40        50        60
PhaC1po         ---------- ---------- ---------D LLTSARMVLA QALKQPFHSA KHVAHFGLEL
PhaC1pp         MSNKNNDELQ RQASENTLGL NPVIGIRRKD LLSSARTVLR QAVRQPLHSA KHVAHFGLEL
PhaC1pm         MRDKSNPAPL PAPATFMNAQ SAVVGVRGRD LLSTMRLLAV QGLKSPVRSS RHLLAFGGQL
PhaC1ls         MSNKNNDELQ RQASENTLGL NPVIGIRRKD LLSSARTVLR QAVRQPLHSA KHVAHFGLEL
PhaC1 16        MAKTKGSRRA RIPSP----- -----PSRSE MLRLALVILG RLRGNPGLPI RHIAGFAKEF
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                70        80        90        100       110       120
PhaC1po         KNVLLGQSAL QPEDGDRRFN DPAWSQNPLY RRYLQTYLAW RKELHDWIEH SSLSEQDASR
PhaC1pp         KNVLLGKSSL APDSDDRRFN DPAWSNNPLY RRYLQTYLAW RKELQDWVSS SDLSPQDISR
PhaC1pm         GRVLLGDTLH KVNPQDARFA DPTWHLNPFY RRSLQAYLAW QKQLSAWIDN SDLSPDDRAR
PhaC1ls         KNVLLGKSSL APDSDDRRFN DPAWSNNPLY RRYLQTYLAW RKELQDWVSS SDLSPQDISR
PhaC1 16        LNILRGKSDV TPAAGDKRFA DAAWSGNRLS GALVQVYIAA SQEAYALVKE LELEGRDASA
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                130       140       150       160       170       180
PhaC1po         GHFVINLMTE AMAPSNS-MA NPAAVKRFFE TGGKSLLDGL SHLAKDMVNN GGMPSQVNME
PhaC1pp         GQFVINLMTE AMAPTNT-LS NPAAVKRFFE TGGKSLLDGL SNLAKDMVNN GGMPSQVNMD
PhaC1pm         ARFLASLMSD ALSPSNS-PL NPQAVKELFN TGGSSLLKGV RHLFDDLLHN NGLPSQVSKH
PhaC1ls         GQFVINLMTE AMAPTNT-LS NPAAVKRFFE TGGKSLLDGL SNLAKDMVNN GGMPSQVNMD
PhaC1 16        ARFLSTMLVE ALAPSNNPLT NPVVLKRVRE TRGKSLLRGF QHFLSDRLDN AGMISMVDRT
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                190       200       210       220       230       240
PhaC1po         AFEVGKNLAT TEGAVVFRND VLELIQYKPI TESVHERPLL VVPPQINKFY VFDLSPDKSL
PhaC1pp         AFEVGKNLGT SEGAVVYRND VLELIQYSPI TEQVHARPLL VVPPQINKFY VFDLSPEKSL
PhaC1pm         AFEVGRNLAC TPGAVVFRNE LLELIQYKPM SERQYLRPLL IVPPQINKYY IFDLSNDKSF
PhaC1ls         AFEVGKNLGT SEGAVVYRND VLELIQYSPI TEQVHARPLL VVPPQINKFY VFDLSPEKSL
PhaC1 16        AFEVGRNVAA TPGDVVFRNE VLELLQYKPQ SAEVYEIPLM VVPPQINKYY VLDLSPGNSF
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                250       260       270       280       290       300
PhaC1po         ARFLLRSQVQ TFVVSWRNPT KAQREWGLST YIEALKDAID VICAITGSKD VNMLGACSGG
PhaC1pp         ARFCLRSQQQ TFIISWRNPT KAQREWGLST YIDALKEAVD AVLSITGSKD LNMLGACSGG
PhaC1pm         VQYALKNGLQ TFMISWRNPD PRHREWGLSS YVQAVEEAIE ACRAISGSKE VNLLGACAGG
PhaC1ls         ARFCLRSQQQ TFIISWRNPT KAQREWGLST YIDALKEAVD AVLSITGSKD LNMLGACSGG
PhaC1 16        VNYAVKQGFQ VFMVSWRNPG PELAHLSLDH YLRGLEEAHN AVMEITGAKK VSFKGLCAGG
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                310       320       330       340       350       360
PhaC1po         LTTASLLGHY AALGQQ-KVN ALTLLVSVLD TQ--LDTQVA LFADEKTLEA AKRRSYQAGV
PhaC1pp         ITCTALVGHY AAIGEN-KVN ALTLLVSVLD TT--MDNQVA LFVDEQTLEA AKRHSYQAGV
PhaC1pm         LTIAALQGHL QARRQLRKVS SATYMVSLLD SQ--IDSPAM LFADEQTLES AKRRSYQQGV
PhaC1ls         ITCTALVGHY AAIGEN-KVN ALTLLVSVLD TT--MDNQVA LFVDEQTLEA AKRHSYQAGV
PhaC1 16        LTSAIALGRY ADQGKLDTVN TLTLNVTLLD ISGMEKTNMG YFLTPEGLET SMKRSKKEGV
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                370       380       390       400       410       420
PhaC1po         LEGSDMAKVF AWMRPNDLIW NYWVNNYLLG NEPPVFDILY WNNDTTRLPA ALHGEFIEMF
PhaC1pp         LEGSEMAKVF AWMRPNDLIW NYWVNNYLLG NEPPVFDILF WNNDTTRLPA AFHGDLIEMF
PhaC1pm         LDGRDMARVF AWMRPNDLIW NYWVNNYLLG KQPPAFDILY WNNDNTRLPA ALHGDLLDFF
PhaC1ls         LEGSEMAKVF AWMRPNDLIW NYWVNNYLLG NEPPVFDILF WNNDTTRLPA AFHGDLIEMF
PhaC1 16        LYGHEMAKMF AWLRPNDLVW NYWVNNYLMG QKPAAFDVLY WNSDSTRLPA ALHHDFCDMV
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                430       440       450       460       470       480
PhaC1po         QTNPLTRPGA LEVCGTPIDL KQVTCDFFVV AGTTDHITPW DSCYKSAHLF GGKCEFVLSN
PhaC1pp         KSNPLTRPDA LEVCGTAIDL KQVKCDIYSL AGTNDHITPW PSCYRSAHLF GGKIEFVLSN
PhaC1pm         KHNPLRRAGG LEVCGTPVDL TKVNVDSFSV AGINDHITPW DAVYRSTLLL GGNRRFVLSN
PhaC1ls         KSNPLTRPDA LEVCGTAIDL KQVKCDIYSL AGTNDHITPW PSCYRSAHLF GGKIEFVLSN
PhaC1 clon      AKNELGNGEV YQLPDAQVDL SRVDVESFIV AGRTDHITPW DACYKSVNLL GGKSQFVLVN
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                490       500       510       520       530       540
PhaC1po         SGHIQSILNP PGNPKARYMT NSEMPLDPKA WQESSTKHAD SWWLHWQTWL AERSGETKNA
PhaC1pp         SGHIQSILNP PGNPKARFMT GADRPGDPVA WQENAIKHAD SWWLHWQSWL GERAGALKKA
PhaC1pm         SGHIQSILNP PGNPKANYYE NAKLTSDPRA WFHDAQHQQG SWWPQWLEWI QARSGEQRET
PhaC1ls         SGHIQSILNP PGNPKARFMT GADRPGDPVA WQENATKHAD SWWLHWQSWL GERAGALKKA
PhaC1 16        SGHIQTLVCP PGKGKASYQT ADVLPEDPHE WLEHSKATTG SWWEMWIKWA SERHGPKKPA
```

Figure 10. Multiple alignment of amino acid sequences of PHA synthases (PhaC1) from *Pseudomonas oleovorans* Gpo1 (PhaC1po), *Pseudomonas putida* KT2440 (PhaC1pp), *Pseudomonas putida* LS46 (PhaC1ls), and $PhaC1_{16}$ using Clustal Omega.

3.7. Predicted 3D Structure of PhaC1 Proteins

As no three-dimensional (3D) structures of PHA synthases (PhaC1 proteins) derived from X-ray crystallography have been published, 3D structures of the *P. putida* LS46 PhaC1 and $PhaC1_{16}$ proteins were predicted using Phyre2 (Figure 11). The Phyre2 program was able to model 303 residues of the *P. putida* LS46 PhaC1 amino acid residues with 99.94% confidence.

The *P. putida* LS46 PhaC1 protein showed 12% sequence identity to gastric and bacterial lipases. In $PhaC1_{16}$, 331 residues were modeled with 99.9% confidence. In the *P. putida* LS46 PhaC1 protein, 44% of the amino acid residues were associated with 21 α-helices and 11% were associated with 12 β-helices, while the $PhaC1_{16}$ protein contained 20 α-helices and 12 β-sheets (Figure 11). His479 and Asp451 were identified at the catalytic site of the *P. putida* LS46 PhaC1 protein, while His473 was identified at the catalytic site in $PhaC1_{16}$, which was identical to the template (gastric lipase).

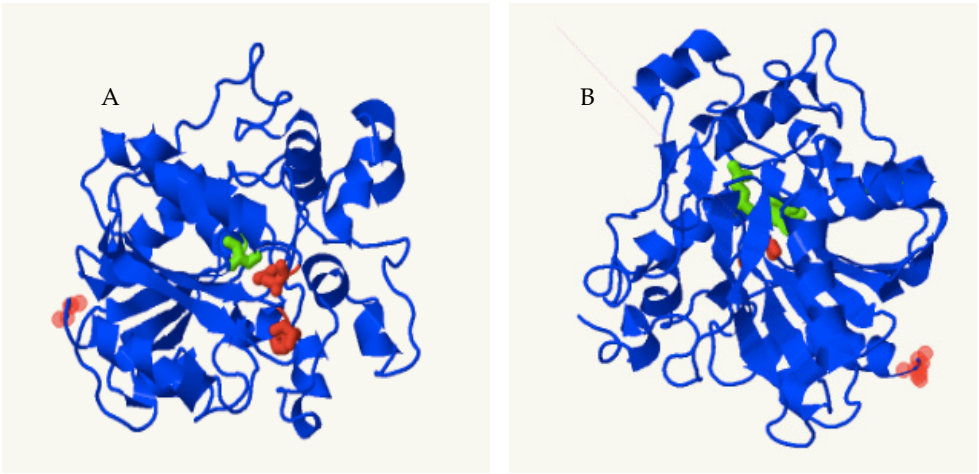

Figure 11. Comparison of predicted 3D structures of proteins encoded by (**A**) *P. putida* LS46 $PhaC1_{ls}$ and (**B**) *PhaC1*$_{16}$. 3D models were constructed with PhaC1 of *P. putida* LS46 (EMR48251), $PhaC1_{16}$ (ALV86417.1) using online Phyre2. Red areas indicate amino acid at putative catalytic sites in the PhaC1 proteins which identical to template. The green area indicates amino acids at the catalytic site, which are different from the template (gastric lipase).

4. Discussion

P. putida LS46 was isolated from wastewater and, like most other *P. putida* strains, can produce only MCL-PHAs [10]. PHA synthase (PhaC1 and PhaC2) enzymes are highly conserved among different strains of *P. putida* [7]. Some *Pseudomonas* species, like *Pseudomonas* sp. 61, *P. mendocina*, *P. extremaustralis*, and *P. pseudoalcaligenes* have been shown to synthesize PHB/PHA co-polymers [41–44]. Recombinants of *P. putida*, *P. oleovorans*, *P. resionovorans*, *P. entomophila*, *P. mendocina*, *P. aeruginosa*, and *P. stutzeri* that synthesize co-polymers of and MCL-PHAs utilizing low cost carbon sources have been reported in the literature [45,46]. Even the carbon:nitrogen (C/N) molar ratio has been reported to affect the subunit composition of PHAs in *P. pseudoalkaligenes* YS1, with higher C/N molar ratios resulting in greater incorporation of medium chain length monomers in the polymers [47].

A number of PhaC1 proteins of Class I PHA synthases have broad substrate specificity and could produce random co-polymers of 3HB and 3HHx. Number of recombinant bacteria that express the *phaC1* gene of *Aeromonas cavaie* have been shown to synthesize 3HB-co-3HHx polymers [48–50]. Mutagenesis of PhaC1 of *Pseudomonas* sp. 61-3 of Glu130 to Asp130 (E130D), Ser325 to Cys325 or Thr325 and Gln481 to Met481, lys481 or Lys481 led to 20%–40% 3HB in copolymer [51]. In $PhaC1_{16}$ only one change identical this to this (Ser325 Thr) mutagenesis was observed. This could be a possible reason for the higher incorporation of 3HB in copolymer.

The substrate specificity of PHA synthases has been modified by PhaC mutagenesis in *Ralstonia eutropha* H16, *A. caviae*, *P. putida* Gpo1, and *Pseudomonas* sp. 61-3 [39,52–54]. Comparison of the amino sequences of $PhaC1_{16}$ protein and other Class I and Class II PHA synthases revealed both low sequence identity and homology with the Class I PHA synthase of *R. eutropha* H16 (38% identity, 54% homology) and with the Class II PHA synthase of *P. putida* LS46 (46% identity, 61% homology). Directed evolution of Class II PHA synthase of *Pseudomonas* sp. 61-3 identified Ser325, Ser447 and Gln481, which are involved in substrate specificity of PHA synthase [55,56]. Site-directed mutagenesis of *phaC1* of *P. putida* KT2440, *P. chlororaphis*, *P. resinovorans*, and *P. aeuginosa* at four residues (Glu130, Ser325, Ser477, and Gln481) had a similar effect on substrate specificity [52]. Multiple sequence alignment showed that the six conserved regions have 23 different amino acids that are naturally involved and associated with the substrate specificity. Random mutagenesis of PHA synthase of *P. putida* led to altered substrate specificity; however, no individual mutation was identified for this change. The PHA synthase of LS46 (PHA_{LS46}) and LS46123 (PHA_{16}) had 15 amino acids different but it is difficult to identify the effect of individual amino acids on the substrate specificity of an enzyme.

Sheu and Lee [40] compared six conserved regions of the PHA synthase of *P. putida* Gpo1 with mutated PHA synthases, which they showed were able to add a higher mol % of 3HB to co-polymers. Further comparison of the amino acid sequences of the mutated PHA synthase from *P. putida* Gpo1 with the $PhaC1_{16}$ revealed 18 amino acid substitutions across the six conserved regions. At least 12 amino acids in $PhaC_{16}$ were different to the amino acids of the *P. putida* LS46 PhaC1. These data indicate that the $PhaC1_{16}$ enzyme was novel and the amino acid sequences in the usually conserved regions of PHA synthase were unique and some changes are identical to known PhaC1 mutations.

Chen et al. [57] reported three mutations (Ala295Val, Ser482Gly, and Leu484Val) in the mutagenized versus wild-type PHA synthase of *P. putida* Gpo1, which were responsible for addition of up to 60% 3HB in co-polymers produced by *P. putida* Gpo1. Comparison of the amino acids in positions 295, 482, and 484, which were identified as key amino acids for substrate specificity, revealed amino acids in $PhaC1_{16}$ that were different from both the *P. putida* LS46 PHA synthase (Leu295Ala, Thr482Ser, and Val484Ile) and the *P. putida* Gpo1 PHA synthase. Three amino acids in the PHA synthase of *Pseudomonas* sp. 61-3, which, when mutated (Ser325, Cys/Ser477, and Lys/Gln481Leu), could add a greater mol % of 3HB into copolymers. The $PhaC1_{16}$ enzyme had amino acids in these positions that were different than those in the *P. putida* LS46 PhaC1 enzyme (Ser325Cys and Gln481Leu) [40,56]. The amino acids in these two positions were also different from those encoded by the *Pseudomonas* sp. 61-3 PHA synthase. This may be the reason for the greater incorporation of 3HB in co-polymer produced by *P. putida* LS46123. Mutations of Cys296 to Ser296 and His453 to Gln453 result in greater incorporation of (R)-3-hydroxyhexanoyl-CoA and (R)-3-hydroxydodecanoyl-CoA into MCL-PHAs, but not SCL-PHA monomers like (R)-3-hydroxybutyryl-CoA [51]. Another important difference in $PhaC1_{16}$ was Leu484 to Val484, which may result in increased 3-hydroxybutyrate content in PHAs. Likewise, transfer of plasmid with single mutations in PhaC1 of *Pseudomonas* sp. 61-3 Q481M and S482G enhanced the (R)-3-hydroxyhexanoate monomer composition in the PHA accumulation by a *P. putida* GPp104 (PHA^-) [56]. Therefore, the amino acid sequence of $PhaC1_{16}$ is novel; it did not match any of the wild-type or mutated PhaC1 sequences. This could be the possible reason for the unique monomer composition of the polymer produced by *P. putida* LS46123.

The monomer composition of PHA polymers greatly influences their physical and thermal properties [8,9]. Unlike the polymers produced by *P. putida* LS46, polymers produced by *P. putida* LS46123 on different substrates were semisolid. Therefore some of the physical property tests used for solid polymers, like Young's modulus and stress strain tests, were not performed. Integration of the different peaks generated by ^{1}HNMR analysis demonstrated that polymers synthesized by *P. putida* LS46123 are co-polymers of 3HB and 3HA. This was further confirmed by FT-IR analysis, which showed the stretching of the carbonyl group. The polymers synthesized by *P. putida* LS46123 contained 40%–50% of 3HB. *P. putida* LS46123 recombinant accumulated higher 3HB in PHAs than a recombinant of *P. putida* KT2440, indicating the role of the host and some other accessary genes

in the fatty acid metabolism pathways [31]. Generally, polymers with high 3HB content have high melting temperature and toughness. The production of such polymers requires wide substrate specificity from the PHA synthase enzyme. *A. caviae*, *Pseudomonas* sp. 61-3, and *P. stutzeri* PHA synthases have been shown to have wide substrate specificity and were used to produce co-polymers of SCL- and MCL-monomers [57,58]. The PhaC1$_{16}$ PHA synthase also displayed wide substrate specificity and could add both SCL- and MCL-monomers to the PHA polymers synthesized. However, unlike optimized PHA synthase of *P. stutzeri*, the PhaC1$_{16}$ enzyme added a greater proportion of 3HHx and 3HO subunits than 3HD subunits, indicating that this PHA synthase had broad substrate specificity [59].

PHA copolymers with different monomer composition and contents are known to have different physical and thermal properties [19,60,61]. Thermal properties such as glass transition temperature (T_g), melting temperature (T_m), and thermal degradation temperature (T_d) are commonly evaluated for PHA polymers to determine the temperature conditions at which the polymer can be processed and utilized. MCL-3HA is added to polymers to lower their crystallinity and melting temperature [62].

The physical and chemical properties of copolymers are largely dependent on monomer composition—i.e., the mol % of 3HV, 3HHx, and 3HO subunits. Copolymers with increasing mol % (24 mol % to 71 mol %) of 3HV display decreasing in melting temperatures (from 177 °C to 87 °C), decreasing glass transition temperatures (from 9.0 °C to −9.0 °C), and elongation-to-break ratios increased by 900 times [11,63]. Melting temperatures and glass transition temperatures of MCL-PHAs produced by *P. putida* LS46 from octanoate, decanoate, or biodiesel-derived fatty acids were 60 °C to 65 °C, and −33 °C to −35 °C, respectively [64].

Generally the ideal polymer should have a melting temperature far below it degradation temperature. DSC showed the different properties of PHAs produced by *P. putida* LS46123. In the present study, co-polymers containing 3HB and 3HV in different proportions with MCL-monomers displayed decreased glass transition (from −34 °C to −36 °C), as well as melting temperatures from 138 °C to 166 °C. It appears that 3HB content is not solely responsible for high melting temperature [64]. PHAs produced on octanoic acid and biodiesel fatty acid had about 50% of 3HB but had a different melting temperature. Melting temperatures were more correlated with 3HHx and 3HV content and PHAs produced on hexanoic acid with 70% 3HHx have low melting temperature than PHAs produced on octanoic acid or decanoic acid, which had 42%–48% 3HHx. However, this it was not true for PHAs produced on biodiesel fatty acid, which had the highest content of 3HO. All our polymers had a very high degradation temperature (248–254 °C), which was much higher than the melting temperature. However, the degradation temperatures of Mirel F1006 (a copolymer of 3HB and 4-HB), Mirel 3002, and the P229 polymer were higher than our polymer [65]. Glass transition temperature is a very important parameter for stability at low temperatures. Glass transition temperature (T_g) varied from 4 to −44 with a change in 3HA content from 0% to 97% in the polymer [19]. Melting temperature (T_m) decreased from 178 °C to 42 °C, with the change proportional to the amount of 3HA in the polymer. Our polymers had T_g and T_m values very similar to LDPE and thus were suitable for wider application.

Complex viscosity is related to shear velocity as a function of shear rate. Complex viscosity of all polymers produced in hexanoic, octanoic, nonanoic, and biodiesel fatty acid decreased with increased angular frequency. This decrease is associated with low melting stability. However, the complex viscosity of our polymers was higher than commercial polymers like Mirel F1006, Mirel 3002, and P229 [65]. Carbon substrates produced for PHAs production not only affected monomer composition but also led to the production of a polymer with diverse physical, thermal, and rheological properties.

5. Conclusions

A new recombinant strain of *P. putida*, designated LS46123, carrying a novel PHA synthase (PhaC1$_{16}$) from a metagenomic library, was able to incorporate SCL- and MCL-monomers into PHA polymers from related and non-related substrates. GC, NMR, and FT-IR confirmed the molecular structures of the PHA polymers. The mixture of SCL-monomers (3HB and 3HV) and MCL-monomers

(3HHx, 3HO, and 3HN) in the polymers greatly influenced both their physical and thermal properties. In this respect, the PhaC1$_{16}$ enzyme was similar to other PHA synthases from *A. caviae*, *P. mendocina*, and *Pseudomonas* sp. 61-3, which can add both SCL- and MCL-monomers to PHAs. However, the PhaC1$_{16}$ synthase expressed by *P. putida* LS46123 contained unique amino acids in key functional regions of the enzyme, and the PHA polymers synthesized by *P. putida* LS46123 consisted of monomer compositions that were unique and different from the PHA polymers synthesized by other bacteria. The presence of 3HB in PHAs produced by *P. putida* LS46123 decreased their glass transition temperatures as well as their melting temperatures. A metagenomic library containing novel PhaC enzymes will lead to the identification of new polymers.

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