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Physiological Characterization and Genetic Engineering of *Pseudomonas corrugata* for Medium-Chain-Length Polyhydroxyalkanoates Synthesis from Triacylglycerols

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Abstract. *Pseudomonas* belonging to the rRNA-DNA homology group I produce medium-chain-length (mcl)-polyhydroxyalkanoates (PHA). We show that *P. corrugata*, a member of this group, accumulates 0.5–1.0 g of mcl-PHA/L of culture when grown on glucose (Gl) or oleic acid (Ol). The predominant monomers of Gl-PHA and Ol-PHA are β-hydroxydecanoate and β-hydroxyoctanoate, respectively. The molecular masses and polydispersity of *P. corrugata* PHAs are higher than those typically found with other *Pseudomonas*. We electrotransformed *P. corrugata* with a plasmid pCN51lip-1 carrying *Pseudomonas* lipase genes to generate strain III111-1. The recombinant strain grew on intact triacylglycerols (TAGs) to 1.9–2.7 g of cell-dry-weight/L of culture. The yields and the predominant repeat-units of PHAs obtained from the lard- and tallow-grown III111-1 were similar to those of Ol-PHA from wild-type cells. In contrast to other *Pseudomonas* species, *P. corrugata* III111-1 grown on TAGs at temperatures up to 36°C was not significantly affected with regard to cell yields, amounts of PHA produced, and the repeat unit compositions of the polymer.

Polyhydroxyalkanoates (PHAs) are biomaterials produced and sequestered in the form of intracellular granules by many bacteria. These biopolymers are usually synthesized when a carbon source is in abundance, but one or more of the other nutrients needed for growth have been exhausted. These biopolymers have material properties similar to those of thermoplastics, elastomers, and adhesives. As such, there is an ongoing interest in developing PHAs as biodegradable counterparts to the petroleum-based polymers [1, 19]. One class of PHA is termed medium-chain-length (mcl)-PHAs, which are mostly synthesized by the rRNA-DNA homology group I *Pseudomonas* and contain β-hydroxyalkanoate repeat units of 6–14 carbon atoms [5, 7, 12]. The physicochemical and materials properties of mcl-PHAs render these polymers potentially useful in the formulation of biodegradable elastomers and adhesives [1].

The genetic loci responsible for the biosynthesis of PHAs have been extensively characterized [19, 27]. Those found in mcl-PHA-synthesizing *Pseudomonas* have been classified as the type II *pha* locus. The three genes that respectively code for PHA polymerase 1 (*phaC1*), PHA depolymerase (*phaZ*), and PHA polymerase 2 (*phaC2*) are arranged in the chromosome in the order of *phaC1-phaZ-phaC2*. We have previously described a rapid and specific PCR-based assay to screen for the type II *pha* locus [25]. Using this assay, we identified *P. corrugata* belonging to the rRNA-DNA homology group I as harboring the type II *pha* gene locus. We have proceeded to verify the synthesis of mcl-PHA by this bacterium grown on glucose or oleic acid [25]. Kessler and Palleroni [16] have independently reported the occurrence of mcl-PHA in *P. corrugata* grown on glucose or octanoate. Unlike the other mcl-PHA-producing *Pseudomonas*, we noted that *P. corrugata* grows well at temperatures as high as 37°C. Since

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intact fats and oils are attractive renewable feedstocks for fermentation, there is an ongoing interest in utilizing these substrates for PHA synthesis [2, 6, 8, 10, 13, 17, 22]. In this respect, the growth-temperature property of *P. corrugata* might be advantageous when fats/oils with high melting points are used. In this communication, we describe the genetic engineering of *P. corrugata* to allow it to use intact TAGs for growth and mcl-PHA synthesis. The properties of the polymers derived from TAG fermentation by the recombinant *P. corrugata* were also characterized.

Materials and Methods

Bacteria, culture conditions, and plasmids. *P. corrugata* 388 was obtained from W. F. Fett (ERRC/ARS/USDA, Wyndmoor, PA). This strain was originally isolated from alfalfa roots by F.L. Lukezic (Pennsylvania State University, University Park, PA). *Escherichia coli* DH5α (Life Technologies, Inc., Gaithersburg, MD) was used in all DNA subcloning and plasmid propagation experiments. Growth media were Luria (1% wt/vol tryptone, 0.5% wt/vol yeast extract, 0.5% NaCl) and tryptic soy broth (TSB; Difco, Detroit, MI). Cultures were routinely grown at 30°C (for the *Pseudomonas*) or 37°C (for *E. coli*) with 200–250 rpm shaking. The solid media were prepared by adding agar (1.2–1.5% wt/vol) to the corresponding broth before autoclaving. When needed, kanamycin (Km, 30–50 μg/ml), ampicillin (Ap, 50–100 μg/ml) and carbenicillin (Cb, 50 μg/ml) were added to the growth media. The pCN51 vector [18] and the pSG312 plasmid containing *Pseudomonas* lipase genes were purchased from the American Type Culture Collection (ATCC; Manassas, VA).

Molecular biology procedure. Restriction endonucleases, Klenow enzyme, calf intestinal alkaline phosphatase, and T4 DNA ligase were obtained from Life Technologies or New England Biolabs, Inc. (Beverly, MA). These enzymes were used according to the instructions provided by the suppliers. Mini plasmid screening was performed as described [20]. For large-scale plasmid preparation, we either followed the method of Sambrook et al. [20] or used a Plasmid Midi Kit (Qiagen, Inc., Valencia, CA). Restriction DNA-fragments were analyzed by agarose gel electrophoresis in TBE buffer system (0.089 M Tris base, 0.089 M boric acid, 0.002 M Na-EDTA).

Construction of pCN51lip-1. Recombinant plasmid pCN51lip-1 was constructed by splicing the *Pseudomonas* lipase genes of pSG312 into the shuttle vector pCN51 [18]. Briefly, pSG312 was digested with *Pvu*II restriction enzyme. A 3.4-kb DNA fragment containing the *Pseudomonas lipA* and *limA* genes that code for a lipase precursor (364 amino acids) and a modulator protein (345 amino acids) [15], respectively, was isolated by agarose gel electrophoresis, extracted with GenElute™ Agarose Spin Column (Supelco Inc., Bellafonte, PA), purified on an Elutip-d® column (Schleicher and Schuell, Keene, NH), and concentrated by ethanol precipitation [20]. This fragment was then ligated by using T4 DNA ligase (New England Biolabs.) to the pCN51 shuttle vector that had been linearized with *Bam*HI restriction digestion, treated with Klenow enzyme (Life Technologies) to fill in the 5′-protruding ends, and reacted with calf intestinal alkaline phosphatase (New England Biolabs) to remove the 5′-phosphate group. The ligation mixture was used to transform *E. coli* DH5α according to the protocol provided by the supplier (Life Technologies). Transformants were selected on Luria broth-agar medium containing Km. Recombinant plasmids isolated from the transformants were characterized by restriction-enzyme analysis. Figure 1 shows the map of the recombinant plasmid, pCN51lip-1, used in this study.

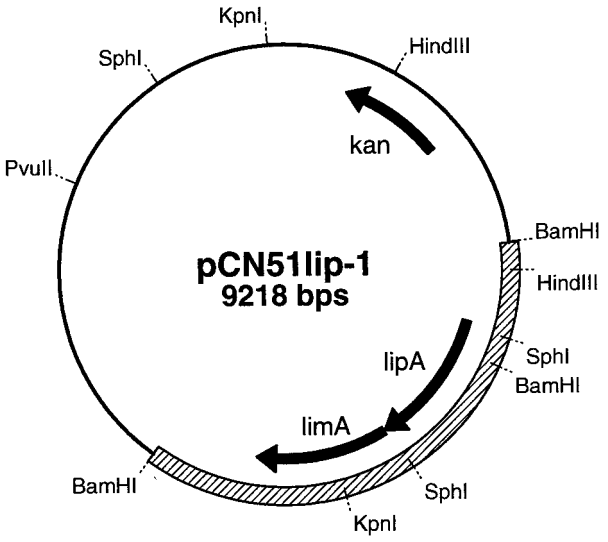

Fig. 1. Map of pCN51lip-1. Sequence of pCN51 vector is represented by bold line, and the *lip* operon-containing fragment of pSG312 by hatched box. Abbreviations: *kan*, Km-resistance gene; *lipA* and *limA*, putative coding sequences of lipase precursor and lipase modulator protein, respectively.

Electroporation. *P. corrugata* 388 was electroporated with pCN51lip-1 according to a previously described method [23]. In short, a seed culture (3 ml) was prepared by growing the target bacterium overnight in TSB medium at 30°C. Two ml of the seed culture was used to inoculate 48 ml of Luria medium in a 125-ml Erlenmeyer flask. The culture was grown for 45 min at 30°C at 250 rpm. Cells (25 ml of culture) were harvested by centrifugation (7,000 *g*, 35 min, 4°C), washed once in prechilled, sterile 0.3 M sucrose solution (5 ml), and resuspended in the same solution to an $O.D._{600nm}$ of 5.0. The concentrated cell suspension (100 μl) was mixed with the transforming plasmid (2–5 μg) in an electroporation cuvette (0.1–cm gap width). After a 5-min incubation on ice, electroporation was performed at 2.5 kV, 25 μF, and 200 Ω settings in a Gene Pulser II electroporator equipped with a Pulse Controller Plus module (Bio-Rad Laboratories, Hercules, CA). Immediately after electroporation, the cell suspension was added to 0.9-ml SOC medium [11] in a 15-ml polypropylene Falcon tube (Becton Dickinson Labware, Lincoln Park, NJ). Cells were incubated in an incubator-shaker (30°C, 250 rpm) for 2 h. Transformants were selected by spreading 200 μl of the cell suspension on Luria solid medium containing Km, followed by overnight incubation at 30°C.

Rhodamine-B plate assay. The assay plates were prepared by autoclaving Luria-agar (1%) medium supplemented with olive oil (3% vol/vol). After cooling to 60°C, rhodamine-B solution (0.2 μm filter-sterilized stock of 0.1%, wt/vol) was mixed with the medium to a final concentration of 0.001% (wt/vol). Kanamycin was included in the medium when necessary. The medium was poured into petri dishes and allowed to solidify. To assay for lipase activity, cultures were streaked on the test plates. After sufficient cell growth had occurred, the plates were viewed and photographed under a long-wavelength (366 nm) UV-light illumination. The presence of lipase activity was indicated by the appearance of fluorescent halos around the bacterial colonies or streaks.

Table 1. Cell yields, and production and molecular masses of mcl-PHA from *P. corrugata*[a]

Strain	Feedstock[b]	Cell yield (g/L)	PHA yield (g/L)	Molecular mass[c]		Polydispersity (M_w/M_n)
				M_n ($\times 10^4$ g/mol)	M_w ($\times 10^4$ g/mol)	
388	Glucose	1.52	0.47	n.d.[d]	n.d.	n.a.[d]
	Oleic acid	1.62	0.99	n.d.	n.d.	n.a.
	Glucose + Oleic acid	1.86	0.91	13.5	63.0	4.6
	Glucose + Oleic acid[e]	1.78	0.94	11.6	60.0	5.2
	Lard	(No growth)				
III111-1	Lard	2.62	1.55	10.3	55.9	5.4
	Soybean oil	1.87	0.65	6.7	28.9	4.3
	Coconut oil	2.73	1.77	7.4	34.3	4.6

[a] Unless otherwise specified, cultures were grown at 30°C with 200–250 rpm rotary-shaking for 2.5–3.5 days.
[b] Growth medium was supplemented with the feedstock (each at 0.5% wt/vol) as the carbon source.
[c] M_n = number-average molecular mass; M_n = weight-average molecular mass.
[d] n.d. = not determined; n.a. = not applicable.
[e] Growth was first carried out at 37°C for 24 h, then 30°C for 48 h.

Isolation and characterization of PHA. Inoculant cultures (3–50 ml) were prepared by growing the bacteria in Luria or TSB medium for one to two overnights. These cultures (1–10 ml) were used to inoculate 0.25–1 L of medium E* [5] prepared in Erlenmeyer flasks (0.5 to 2-L capacity). The chemically defined E* medium was supplemented with 0.5% wt/vol of a carbon source and, if necessary, Km (30–50 μg/ml). The cells were grown in a shaker-incubator at 30–37°C at 200–250 rpm for 48–96 h. Cells were collected by centrifugation (7000 *g*, 15–30 min, 4°C), frozen, and lyophilized to dryness. The weight of the dried cells was recorded as cell-dry-weight (CDW). To isolate the PHA, cells were resuspended in excess chloroform and shaken vigorously overnight in a shaker-incubator at ambient room temperature. After removing the cell debris by filtration through a Whatman No. 1 filter paper, the clear chloroform extract was rotoevaporated to dryness. The weight of the residue was recorded and used in the calculation of PHA yields. If further purification was needed, the PHA was redissolved in a minimal amount of chloroform and reprecipitated by drop-wise addition into prechilled methanol with constant stirring. The PHA precipitate was scraped from the stirring bar and beaker into a clean container, dried under vacuum overnight, and stored under nitrogen. In the event of poor PHA precipitation, the solvent was evaporated to dryness by blowing a stream of nitrogen over the sample. The repeat-unit composition of PHA was determined by gas chromatography/mass spectrometric (GC/MS) analysis as previously described [3]. The molecular masses of the polymers were determined by gel permeation chromatography as described by Cromwick et al. [6].

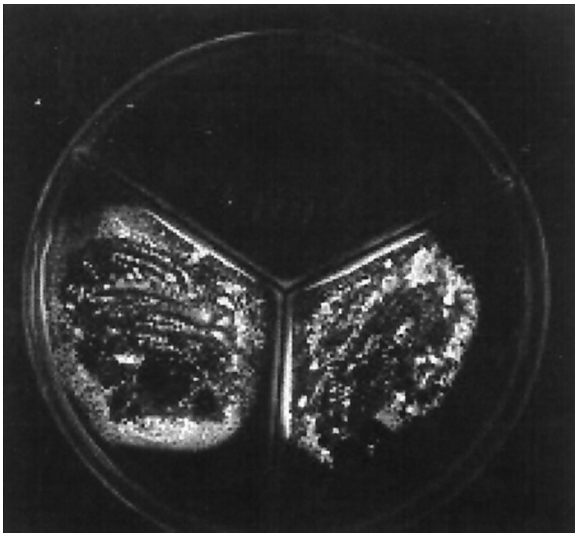

Fig. 2. Plate assay for lipase activity. Stationary-phase cultures of *P. corrugata* were spread on the assay plate. After a 3-day incubation at 30°C, the plate was photographed with the lid removed and long-wavelength UV light illumination from above. *P. corrugata* 388 wild-type, 0°-sector; two randomly chosen Km-resistant, [pCN51lip-1]-containing *P. corrugata* transformants, 135°- and 225°-sectors.

Results

Production and characterization of mcl-PHAs from *P. corrugata*. The ability of *P. corrugata* 388 to grow and produce mcl-PHA by assimilating the commonly used substrates [glucose (Gl) and oleic acid (Ol)] and a representative TAG (lard, La) was first investigated. Table 1 shows that Gl and Ol, alone or together, were fully capable of supporting the growth of *P. corrugata*. Cell yields ranging from 1.52 to 1.86 g cell-dry-weight/L culture were reached after a 3-day growth. In contrast, no cell growth was apparent with La as the sole carbon substrate, suggesting that *P. corrugata* 388 could not hydrolyze intact TAG into its components for subsequent metabolic assimilation. When the organism was assayed for extracellular lipase activity by a rhodamine-B plate method, the result showed that the bacterium did not produce the enzyme (Fig. 2). This observation explained in part the inability of the cells to grow with La as substrate.

Cells grown on Gl and Ol were extracted with chloroform to isolate the PHAs for characterization. Table 1 shows that cells grown on Ol yielded larger amounts of

Table 2. β-Hydroxyalkanoate repeat-units of PHAs from *P. corrugata*

Strain	Feedstock	PHA composition (mol-%)							
		$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{12:1}$	$C_{14:0}$	$C_{14:1}$	$C_{14:2}$
388	Glucose	2	19	56	11	n.d.[b]	2	9	n.d.
	Oleic acid	5	37	33	12	n.d.	2	12	n.d.
	Oleic acid[a]	5	35	31	11	n.d.	2	16	n.d.
	Glucose + Oleic acid	5	48	24	6	n.d.	n.d.	16	n.d.
	Glucose + Oleic acid[a]	4	44	24	6	n.d.	n.d.	21	n.d.
	Glucose + Oleic acid[a,c]	2	33	27	9	n.d.	2	26	n.d.
III111-1	Lard	3	42	28	8	4	2	9	6
	Soybean oil[d]	3	48	23	4	1	1	5	13
	Coconut oil	3	42	35	17	n.d.	3	1	n.d.

[a] Residues obtained after complete removal of the solvent from chloroform extracts of cells were directly processed for GC/MS analysis.
[b] n.d. = not detected.
[c] Growth was first carried out at 37°C for 24 h, then 30°C for 48 h.
[d] $C_{14:3}$ (3 mol %) was also found in soybean oil-derived PHA.

crude PHA than cells grown on Gl did. In fact, an attempt to purify the G1-derived PHA by re-precipitation was unsuccessful. In such case, characterization of the PHA was carried out with the crude material. Data in Table 2 show that β-hydroxydecanoate constitutes more than half of the repeat-units of the Gl-derived PHA. In contrast, both the O1- and Gl + Ol-derived *P. corrugata* PHAs contained β-hydroxyoctanoate (33–48 mol-%) and β-hydroxydecanoate (24–33 mol-%) as the major repeat-unit monomers. The data also showed that the repeat-unit composition analysis performed with crude PHA and with methanol-precipitated material gave comparable results, suggesting that both methods of PHA isolation could be used for compositional analysis. The effect of up-shifting the incubation temperature to 37°C for 24 h at the beginning of the growth did not seem to appreciably alter the repeat-unit composition of the Gl + Ol-derived PHA (Table 2).

The molecular masses of *P. corrugata* mcl-PHA were also measured. The results show that the Gl + Ol-derived polymers assumed M_n and M_w values of 13.5×10^4 and 63.0×10^4 g/mol, respectively (Table 1). An initial 24-h shift-up of the growth temperature to 37°C did not drastically change these values.

Construction of *P. corrugata* III111-1. We described in the preceding section that *P. corrugata* 388 lacked an extracellular TAG-hydrolyzing activity and was unable to grow by using intact TAG (i.e., La) as the sole carbon source. To genetically engineer the bacterium to utilize TAG substrates, we electroporated *P. corrugata* 388 with the recombinant plasmid pCN51lip-1 that carries *Pseudomonas lipA* and *limA* genes. Kanamycin-resistant transformants were verified to contain pCN51lip-1 by plasmid isolation and subsequent restriction analysis (data not shown). Two randomly selected pCN51lip-1-containing clones were assayed for the expression of extracellular TAG-hydrolyzing activity by using a rhodamine-B plate method (see Materials and Methods). The results showed that under UV-illumination an intense fluorescence was observed around the transformant cells, indicating that the genetically engineered *P. corrugata* expressed extracellular TAG-metabolizing activity (Fig. 2). Another randomly selected clone, designated as *P. corrugata* III111-1, was subsequently used for study on TAG fermentation to produce PHA.

Synthesis and characterization of PHA from *P. corrugata* III111-1. The ability of the recombinant *P. corrugata* III111-1 to utilize intact TAGs, such as La, soybean oil (So), and coconut oil (Co) as the sole carbon source for cell growth and PHA synthesis was examined. The results showed that the bacterium grew well on these substrates, producing cell yields of 1.87–2.73 g CDW/L of culture. The yields of the PHA extracted from cells grown on TAG ranged from 0.65 to 1.77 g/L depending on the substrate used. Repeat-unit composition analyses showed that β-hydroxyoctanoate (42–48 mol-%) and β-hydroxydecanoate (23–35 mol-%) were the prominent monomers of the PHAs (Table 2). Furthermore, the mcl-PHA isolated from cells grown on So contained a high level of unsaturated monomers (*ca*. 21 mol-%). In contrast, the cells grown on Co accumulated only saturated mcl-PHA. The molecular masses of these TAG-derived PHAs were determined. Data in Table 1 show that their number-average molecular weights ranged from 6.7×10^4 to 10.3×10^4 g/mol with polydispersity values of 4.3–5.4.

Table 3. Effect of temperature on *P. corrugata* III111-1 cell growth and PHA production[a]

Temperature (°C)	Cell yield (g/L)	PHA yield (g/L)	PHA composition (mol %)[b]		
			$C_{8:0}$	$C_{10:0}$	$C_{12:0}$
30	2.7	1.4	45	36	14
32	2.3	0.7	58	31	8
34	2.2	0.9	52	35	10
36	2.3	0.8	60	29	6

[a] Cultures were grown for 3 days with 250-rpm shaking in E* medium supplemented with coconut oil as carbon source.
[b] Only the repeat units constituting >5 mol-% of the PHA are shown. $C_{8:0}$ = β-hydroxyoctanoate; $C_{10:0}$ = β-hydroxydecanoate; $C_{12:0}$ = β-hydroxydodecanoate.

Effect of temperature on *P. corrugata* III111-1. Cell growth and mcl-PHA production by the recombinant *P. corrugata* at several temperatures was studied with Co as substrate. As expected for *P. corrugata*, a growth temperature as high as 37°C did not decrease the final CDW yields. Data in Table 3 show that the transformant grew to cell yields of 2.2–2.7 g of CDW/L of culture regardless of the incubation temperature. Furthermore, the crude PHA yields did not vary significantly over the range of temperatures used (Table 3). However, when the crude PHA preparations were further purified by cold-methanol precipitation, the samples from higher temperature-grown cells consistently produced lesser amounts of the polymer. Compositional analysis of the purified PHA samples showed that they contained β-hydroxyoctanoate and β-hydroxydecanoate as the dominant repeat units (Table 3).

Discussion

Medium-chain-length PHA is produced mainly by the rRNA-DNA homology group I *Pseudomonas* that contain the type II *pha* gene locus [26]. Although *P. corrugata* belongs to this homology group, it was previously classified as a PHB-synthesizing organism based on staining by lipophilic dyes or the occurrence of intracellular granules [21, 28, 30]. Recently, we showed by PCR screening that *P. corrugata* 338 contained the PHA synthase 2 gene (*phaC2*) of the type II *pha* locus [25]. In that study, we isolated and characterized mcl-PHA produced by the bacterium [25]. Kessler and Palleroni [16] independently reported the synthesis of mcl-PHA by *P. corrugata* grown on octanoate or glucose.

The repeat-unit compositions of mcl-PHAs produced by *P. corrugata* varied with the feedstock type. When glucose was used as the substrate, β-hydroxydecanoate was the predominant repeat-unit, whereas, with oleic acid as feedstock, β-hydroxyoctanoate was the more abundant repeat-unit (Table 2). This observation was corroborated by the results of Kessler and Palleroni [16]. Similar to the fatty acid-derived materials, PHA obtained from *P. corrugata* grown on glucose/oleic acid mixed substrate contained β-hydroxyoctanoate as the principal constituent. Apparently, when presented with unrelated and related carbon sources, the organism preferentially uses the intermediates of fatty acid β-oxidation over those of the *de novo* fatty acid synthesis for mcl-PHA synthesis [29]. In contrast to *P. corrugata*, we have observed in a separate study that *P. resinovorans* NRRL B-2649 synthesized mcl-PHA containing β-hydroxydecanoate as the most abundant repeat-unit monomer regardless of the use of glucose or/and triacylglycerol as substrates [4].

Many studies have documented the synthesis of mcl-PHA in a large number of *Pseudomonas* [26], but only relatively few have reported the use of intact triacylglycerols for cell growth and mcl-PHA synthesis [6, 8, 13, 24]. Like most mcl-PHA-accumulating *Pseudomonas*, we found that *P. corrugata* 388 also failed to grow on lard, an intact TAG (Table 1). Accordingly, we genetically engineered the bacterium to yield strain III111-1, which exhibited growth and PHA-synthesis on TAGs. The cell yield and PHA yields obtained with the genetically constructed strain were comparable to those produced by wild-type strain. The molecular masses of So- and Co-derived PHAs, however, were slightly lower than those of La-derived PHA and the wild-type mcl-PHAs. Since a variety of culture conditions could affect the molecular weight of PHA [14], further study is needed to delineate the observation seen here. The repeat-unit compositions of the TAG-derived PHAs conform to the expected pattern reported earlier [2]. For example, soybean oil, having a high content of mono- and poly-unsaturated fatty acids, yielded a PHA containing a high proportion of unsaturated repeat units. Conversely, the use of coconut oil as substrate resulted in the production of a PHA with saturated repeat-units.

The effect of growth temperature on PHA synthesis in *P. putida* was examined previously [14]. In that study, the rate of PHA synthesis was shown to increase up to

30°C, and cell lysis was observed at 35°C. Many *Pseudomonas* that produce PHA are mesophiles with optimal growth temperature at 30°C and are expected to behave similarly to *P. putida*. Our results with *P. corrugata* III111-1 showed that, unlike *P. putida* and possibly other mcl-PHA-producing mesophilic *Pseudomonas*, cell growth and crude PHA synthesis were not markedly affected at growth temperatures as high as 36°C (Table 3). Furthermore, the growth temperature did not significantly alter the repeat-unit composition of the crude PHA polymers. We observed, however, that crude PHAs obtained at higher temperatures yielded less purified PHA by cold-methanol precipitation. Structural differences not identified in this study might play a role in the precipitability of the polymers. Alternatively, the crude product might contain non-PHA materials that did not co-precipitate with pure PHA. Further study is needed in this respect.

The recombinant *P. corrugata* constructed and characterized in this study is potentially useful for the production of mcl-PHA under certain conditions. For example, in contrast to the other mcl-PHA-producing strains, its ability to grow and synthesize PHA at an elevated temperature could facilitate a more efficient utilization of the fat substrates. In addition, *P. corrugata* is known to synthesize exopolysaccharides, such as alginates [9]. Simultaneous production of mcl-PHA and alginates could be an attractive approach to address the high costs of these biomaterials.

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