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Production and characterization of medium-chain-length polyhydroxyalkanoates by *Pseudomonas mosselii* TO7

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The polyhydroxyalkanoate (PHA) production and growth of *Pseudomonas mosselii* TO7, a newly isolated *Pseudomonas* species from the wastewater of a vegetable oil manufacturing facility, was analyzed. Phenotypic analysis and phylogenetic analysis of the 16S rRNA gene revealed that it is closely related to *Pseudomonas mosselii*. In the presence of palm kernel and soybean oils, *P. mosselii* TO7 produced up to 50% cell dry weight (CDW) medium-chain-length (MCL) PHAs comprising high poly(3-hydroxyoctanoate) (P(3HO)) content; P(3HO) content increased to 45% CDW when grown in octanoate using a single-step culture process. The PHA monomer was identified by ^{13}C nuclear magnetic resonance spectroscopy. The average molecular weight and polydispersity index of PHA were 218.30 ± 31.73 and 2.21 ± 0.18, respectively. The PHA produced by *P. mosselii* TO7 in the presence of palm kernel oil had two melting temperature (T_m) values of 37.2°C and 55.7°C with melting enthalpy (ΔH_m) values of 51.09 J g^{-1} and 26.57 J g^{-1}, respectively. Inhibition analyses using acrylic and 2-bromooctanoic acids revealed β-oxidation as the primary pathway for MCL-PHA biosynthesis using octanoic acid. Moreover, *Pseudomonas putida* GPp104 PHA$^-$, harboring the PHA synthase genes of *P. mosselii* (*phaC1pm* and *phaC2pm*) was used for heterologous expression, which demonstrated that *phaC1pm* is the main PHA synthesis enzyme, and 3-hydroxyoctanoyl-CoA is its major substrate. This was the first report of a *P. mosselii* TO7 isolate producing high-yield P(3HO) through utilization of plant oils.

[**Key words:** Carbon/metabolism; Fatty acids; Plant oils; Polyhydroxyalkanoates; *Pseudomonas mosselii*]

Polyhydroxyalkanoates (PHAs), one of the largest groups of thermoplastic polyesters, consist of biopolymers composed of (*R*)-3-hydroxy fatty acid monomers that are synthesized by various bacteria for carbon and energy storage by PHA synthases (1,2). PHAs are classified as short-chain-length and medium-chain-length PHAs (SCL-PHAs and MCL-PHAs, respectively), depending if the monomers contain 3–5 or 6–14 carbon atoms, respectively (3). While SCL-PHAs are brittle and stiff, MCL-PHAs possess greater elasticity and are more biocompatible (4,5). Given that PHAs are biodegradable, they have the potential to be used as a substitute for non-degradable plastics in packaging materials, agriculture, and biomedicine (6–8). Specifically, the flexible nature of poly (3-hydroxyoctanoate) (P(3HO)) makes it a potentially suitable candidate as a biomaterial for soft tissue engineering and drug delivery (9–11). In addition, PHAs and their composites have been extensively used in medical devices, including but not limited to stents, sutures, repair/regeneration devices, and wound dressings (12). However, studies on MCL-PHAs are rare because large quantities of these polymers are often unavailable due to their high cost of production.

Cupriavidus necator is the most commonly used PHA producer; the most frequently studied MCL-PHA producers include *Pseudomonas putida*, *P. oleovorans*, and *P. mendocina*. However, the monomers produced differ between *Pseudomonas* strains. For example, culture of *P. putida* IPT046 on rice oil produced 61.8% cell dry weight (%CDW) of PHA, which was primarily 3-hydoxydecanoate (3-HD); however, only 19.6%CDW of PHA was observed in *Pseudomonas aeruginosa* IPT171 under the same culture conditions, which was primarily P(3HO) (3,13).

Because the high price of PHA production (i.e., costly substrate) is the major drawback for their use in various new applications and replacement of petroleum-derived plastics, the potential of inexpensive carbon sources are being explored (3). For example, a genetically engineered *P. putida* KT2440 that expressed xylose isomerase (XylA) and xylulokinase (XylB) from *Escherichia coli* W3110 was able to synthesize MCL-PHAs from xylose and octanoic acid (14). In addition, production of PHA from pyrolysis of waste polystyrene by *P. putida* CA-3 has been reported (15). Furthermore, plant oils (e.g., palm and soybean oils) are considered desirable, renewable feedstocks for PHA production given their high carbon content and low cost (16–18).

The objective of this study was to characterize the PHA production and growth of *Pseudomonas mosselii* TO7, a newly isolated *Pseudomonas* species from the effluent of a vegetable oil manufacturing facility. After culturing in the presence of different carbon sources (i.e., fatty acids, carbohydrates and plant oils), the polymer composition and yield of PHA were determined. In addition, the physical properties of the PHA produced by *P. mosselii* TO7 were examined. Furthermore, inhibition analyses were undertaken

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using octanoic acid and gluconate to identify the PHA synthesis pathway, and the PHA polymerase gene of *P. mosselii* was isolated for subsequent genetic engineering in *P. putida* GPp104 PHA$^-$. Identification and characterization of the new *P. mosselii* TO7 may lead to more cost-efficient production of PHA and therefore widen its industrial use.

MATERIALS AND METHODS

Isolation and identification of PHA-producing bacterium Samples were collected from wastewater in a vegetable oil manufacturing factory in Taiwan. The bacterial strains were isolated as described previously (19) with minor modification. Samples were incubated on mineral salt (MS) agar plates (20), containing 0.5% (v/v) octanoic acid as the sole carbon source and cycloheximide (0.06 g/L). The growing colonies were transferred to a new MS agar plate and incubated at 30°C for 72 h to detect accumulation of PHAs by staining colonies with Nile red (0.5 mg/L) (21). The isolated strains that produced PHAs were sub-cultured in flasks containing MS liquid medium with 0.5% (v/v) octanoic acid. The culture was shaken at 200 rpm for 72 h at 30°C to accumulate PHA for its subsequent analysis.

The isolated strain, TO7, was grown at 30°C in 2×YT medium (16 g of Tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) with shaking at 200 rpm in an orbital shaker (model 703R, Hotech, Taiwan).

The 16S rRNA gene of the TO7 cells was amplified by PCR (ABI 9700, Perkin–Elmer Applied Biosystems, Carlsbad, CA, USA) using the prokaryotic 16S rRNA universal primers: pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) (22) under the following conditions: one cycle at 95°C for 5 min; 30 cycles at 94°C for 50 s, 60°C for 50 s, 72°C for 90 s; and one cycle at 72°C for 7 min.

PHA production, extraction, and analysis A one-step culture process was used for production of PHA by TO7 using different carbon sources, including gluconate, fructose, sucrose, hexanoic acid, octanoic acid, decanoate, dodecanoate (Sigma, St. Louis, MO, USA), soybean oil (Uni-President, Tainan, Taiwan) and palm kernel oil (Chant Oil Co., Ltd., New Taipei City, Taiwan). Palm kernel oil was derived from the nut of *Elaeis guineensis* fruit. It includes the triglycerides of the following fatty acids: 48.5% lauric acid (C12:0), 16.2% myristic acid (C14:0), 15.7% oleic acid (C18:1), 3.9% caprylic acid (C8:0), 3.5% capric acid (C10:0), 7.5% palmitic acid (C16:0), 2.6% stearic acid (C18:0) and 2.1% linoleic acid (C18:2) (23). The fatty acid profile of soybean oil contained the following: 54.8% linoleic acid (C18:2), 22.7% oleic acid (C18:1), 10.2% palmitic acid (C16:0), 7.8% α-linolenic acid (C18:3), 4.5% stearic acid (C18:0) and 0.3% arachidic acid (C20:1) (24).

Briefly, bacteria were cultivated in 30 mL MS medium supplemented with a carbon source and incubated at 28°C and 200 rpm for 68 h to accumulate PHA (20). Cells were harvested by centrifugation, washed with a mixture of cold distilled water and cold hexane for removal of residual palm kernel oil (25), and then lyophilized overnight after which %CDW was determined. Approximately 10 mg of the lyophilized cells underwent methanolysis in a solution of 0.85 mL methanol, 0.15 mL 98% sulfuric acid, 2 mg benzoic acid, and 1 mL chloroform at 100°C for 140 min for measurement of PHA content and polymer composition (26). The methylated monomer was determined by gas chromatography as previously described (20).

PHA chemical structure and molecular weight analysis PHA was extracted from 40 mg of lyophilized cells as described elsewhere (27,28). Briefly, PHA was extracted from 40 mg of lyophilized cells in 20 mL chloroform for 12 h with continuous stirring. The mixture was then filtered, and the polymer was precipitated in 10 volumes of chilled methanol for 24 h. The polymer was dried using a rotary vacuum evaporator SPD111V (Thermo, Waltham, MA, USA). PHA chemical structure and molecular weight was determined using ^{13}C nuclear magnetic resonance spectroscopy (NMR) and gel permeation chromatography (GPC), respectively. PHA samples were dissolved in deuterated chloroform ($CDCl_3$ 35 mg/L), and the 125-MHz ^{13}C NMR spectrum was determined using a Bruker Avance 500 NMR spectrometer (Bruker, Coventry, UK) at a probe temperature of 25°C. The chemical shifts were reported in ppm.

A PHA sample of 2.5 mg was dissolved in 1 mL tetrahydrofuran. The injection volume was 0.1 mL, and it was eluted at a flow rate of 1.0 mL/min at 40°C. Six polystyrene molecular weight standards (American Polymer Standards Co., Mentor, OH, USA), with molecular weights ranging from 104 to 382,100 g/mol, were used for calibration. The GPC system was equipped with two Phenomenex columns (Phenogel 5U M2 New Column 300 × 7.8 mm and Phenogel 5U 10^4 Å New Column 300 × 7.8 mm, Phenomenex, Torrance, CA, USA), and a differential refractometer (model T60A, Viscotek Co. Houston, TX, USA) was used to detect the eluted polymer.

PHA purity was determined as previously described (29). Briefly, aliquots of lyophilized bacteria were used to analyze PHA content using gas chromatography to estimate the weight of PHA (A) as well as PHA extraction and weighing (B). The purity of the PHA was 92.30 ± 2.45%, which was determined using the following equation: (A/B) × 100%.

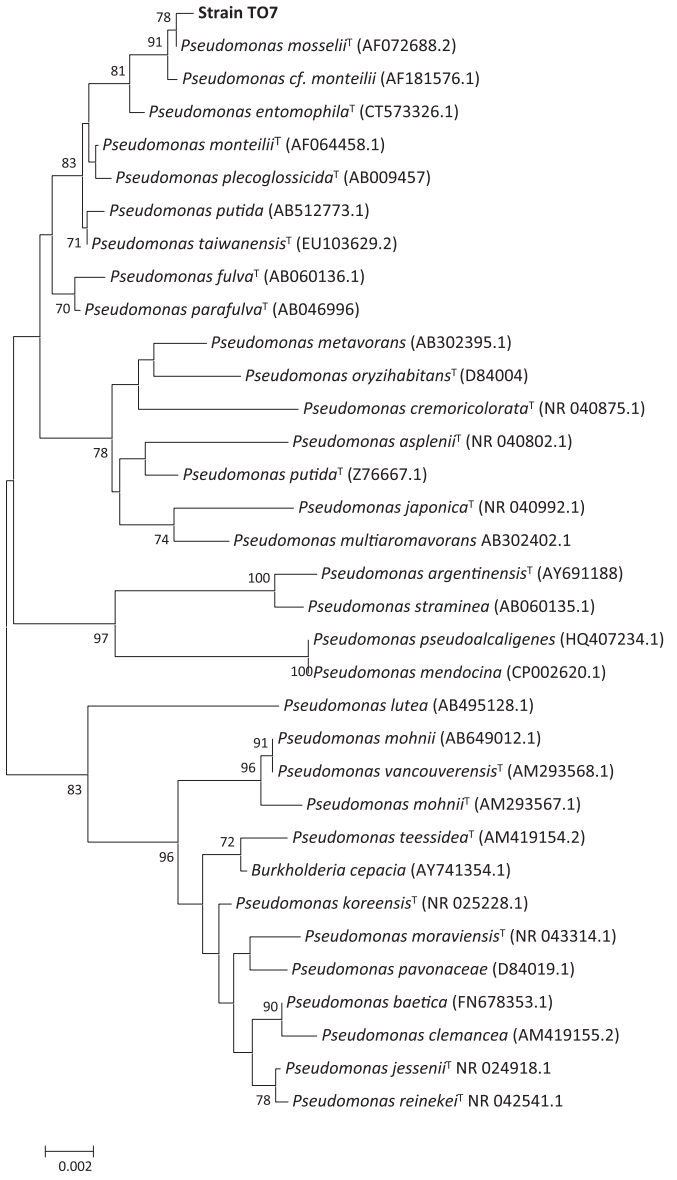

FIG. 1. Isolation and identification of *P. mosselii* TO7. A phylogenetic tree based on the *16S rRNA* nucleotide sequences of *P. mosselii* TO7 and the sequences of neighbors was generated using the neighbor-joining method to illustrate evolutionary relationships. The distance was calculated using the Kimura 2-Parameter algorithm. The sequence of *P. mosselii* TO7 was deposited in GenBank under accession number JQ779914. The number on the branch indicated the bootstrap value over 70% (1000 replicates). The scale bar indicates the number of substitutions per nucleotide position.

Thermal property analysis of PHA After PHA was extracted from 40 mg of lyophilized cells as previously described, its thermal properties were evaluated using differential scanning calorimetry (DSC) analysis using a Perkin Elmer Pyris 6 (Perkin–Elmer, Norwalk, CT, USA) under a nitrogen atmosphere. After a 5 mg sample was encapsulated in an aluminum pan, the temperature range was scanned from −100°C to 200°C with a heating rate of 10°C/min. The DSC endothermal peak values and areas of the second scan were used to evaluate the thermal properties of PHA, including glass transition temperature (T_g), melting temperature (T_m), and melting enthalpy (ΔH_m).

Inhibition experiments A two-step culture process was used for the inhibition experiments to observe the accumulation of PHA by TO7 cells. Bacteria were first cultured in 30 mL 2×YT broth at 30°C for 14 h and then washed with 20 mL MS medium. Various concentrations of acrylic acid and 2-bromooctanoic acid (Sigma, St. Louis, MO, USA) were added after the cells were transferred to 30 mL of MS medium (pH 9) containing 2% (w/v) gluconate and 0.5% (v/v) octanoic acid. The bacteria were cultured for an additional 30 h at 28°C and 200 rpm.

PHA polymerase gene cloning The PHA polymerase genes, *phaC1pm and phaC2pm*, were amplified by PCR using the following primer pairs: 5′-