J. Microbiol. Biotechnol. (2017), 27(5), 990–994
https://doi.org/10.4014/jmb.1612.12031

jmb

Production of Polyhydroxyalkanoates from Sludge Palm Oil Using *Pseudomonas putida* S12

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Received: December 20, 2016
Revised: March 7, 2017
Accepted: March 9, 2017

First published online
March 9, 2017

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pISSN 1017-7825, eISSN 1738-8872

Polyhydroxyalkanoates (PHAs) are biodegradable plastics produced by bacteria, but their use in diverse applications is prohibited by high production costs. To reduce these costs, the conversion by *Pseudomonas* strains of PHAs from crude sludge palm oil (SPO) as an inexpensive renewable raw material was tested. *Pseudomonas putida* S12 was found to produce the highest yield (~41%) of elastomeric medium-chain-length (MCL)-PHAs from SPO. The MCL-PHA characteristics were analyzed by gas-chromatography/mass spectrometry, gel permeation chromatography, and differential scanning calorimetry. These findings may contribute to more widespread use of PHAs by reducing PHA production costs.

Keywords: Sludge palm oil, waste utilization, PHA, *Pseudomonas*, bioplastic

Polyhydroxyalkanoates (PHAs) are biobased polyesters that accumulate in numerous bacteria as an intracellular carbon compound. They are generally classified into two groups: short-chain-length (SCL)-PHAs and medium-chain-length (MCL)-PHAs [1]. SCL-PHAs are stiff and exhibit high tolerance for heat, whereas MCL-PHAs are elastomeric polymers. PHAs are eco-friendly bio plastics that are attractive in terms of biocompatibility [2]. Moreover, PHAs are highly similar to widely used synthetic plastics and can therefore be substituted for petrochemical-based plastics. For these reasons, PHAs have been used commercially in many countries as packaging, storage materials, biofuels, and particularly in biomedical applications [1, 3, 4]. However, impediments exist to the widespread use of PHAs, the largest of which is the high cost of commercial PHA production (2–4 $/kg; depends on substrates, products, process, and reaction scale) [5, 6]. Carbon sources account for approximately 30% of the total operating expenses of PHA production [7]. To solve this problem, inexpensive carbon resources, including renewable wastes such as waste frying oil, olive oil mill waste, bagasse, rice bran, and wheat bran, have been investigated as potential raw materials for PHA production [8, 9]. In addition to these inexpensive carbon resources, low-quality sludge palm oil (SPO) from palm oil mill effluent (POME) represents an attractive potential substrate owing to the continuous increase in palm oil production, which has generated an abundance of SPO [10]. The average annual production of SPO from palm oil processing is 1.5 million tons [11]. In addition, because SPO is the waste product of

the palm oil production process, it can be acquired at little to no cost. Therefore, SPO, whose major components are fatty acids, is a promising alternative feedstock for bacteria, which take up fatty acids well. Additionally, long-chain fatty acids are suitable to make MCL-PHAs [12]; thus, SPO is suitable for use as a substrate. Furthermore, the conversion of SPO to biopolyesters would solve the problem of disposing of waste SPO, offering a more eco-friendly alternative [13]. *Pseudomonas* bacteria possess a beta-oxidation pathway, in which the fatty acids are degraded to remove a C2 acetyl-CoA by FadABDE for each cycle, and can thus utilize fatty acids metabolically [14, 15]. Indeed, some *Pseudomonas* strains have been isolated from POME [2]. In this study, we assessed the abilities of 10 *Pseudomonas* strains to convert SPO to PHAs and characterized the resulting PHAs.

Low-quality crude SPO with an acid value of approximately 120 was obtained from Teck Guan Holdins SDN BHD (Malaysia). The SPO contained approximately 60% free fatty acids and was a good feedstock for the production of PHAs. Because the fatty acid composition of the substrate SPO is important to maintain the quality of the PHAs in PHA production, it was analyzed by gas chromatography. As shown in Table 1, palmitic acid (C16:0) and oleic acid (C18:1) accounted for the majority of fatty acids in the SPO, whereas stearic acid (C18:0), linoleic acid (C18:2), and other fatty acids were less prevalent. This fatty acid composition is similar to that of SPO previously analyzed by other research groups [16].

To determine which *Pseudomonas* strain produced the highest PHA from SPO, 10 strains were cultured in 50 ml of E* medium (0.5 g of NH_4Cl, 5.8 g of K_2HPO_4, 3.7 g of KH_2PO_4, 0.37 g of $MgSO_4$, and 3 ml of trace element solution per liter, pH 7.0) containing 10 g/l SPO as the sole carbon source, at 30°C and 200 rpm for 48 h [7]. To analyze PHAs produced from SPO by *Pseudomonas* strains, impurities in the harvested cells were removed and the cells were then lyophilized by speed vacuum concentration. Next, 20 mg of the dried bacterial biomass was completely dissolved in 0.5 ml of chloroform by vortexing. To methylate the PHAs, 0.5 ml of a solution containing 15% (v/v) H_2SO_4, 85% (v/v) methanol, and 0.2% benzoic acid (as an internal standard) was added to each chloroform solution. The solutions were vortexed and kept in a water bath at 80°C for 3 h. Subsequently, 0.5 ml of distilled water was added, and the solutions were vortexed vigorously. The PHA-rich chloroform was recovered for analysis [17–19]. The PHA composition was analyzed by gas-chromatography/mass spectrometry apparatus (Agilent 7890B, USA) equipped with an HP-5 capillary column (30 m × 0.320 mm) with 0.25 μm film [17]. The compositions of analyzed PHAs were determined based on the methyl-3-hydroxyalkanoates in the Agilent database.

When SPO was used as the sole carbon source, *P. putida* exhibited the highest dry cell weight (DCW). The four strains showing high MCL-PHA production were *Pseudomonas moorei*, *Pseudomonas mohnii*, *Pseudomonas putida* KT2440, and *Pseudomonas putida* S12. MCL-PHA accounted for 20.2%, 21.6%, 20.8%, and 24.9% of the DCW in these strains, respectively (Table 2). It was reported that MCL-PHA productivity is dependent on PHA precursors from the fatty acid β-oxidation metabolism [20]. Therefore, the activity of the enzymes of β-oxidation in *Pseudomonas* species may affect the fatty acid utilization for PHA synthesis [21, 22]. As shown in Fig. 1, MCL-PHAs from the four strains were composed of several types of monomers ranging from C6 to C14, with few differences in monomer

Table 1. Fatty acid composition of sludge palm oil (SPO), as analyzed by gas chromatography (GC).

Fatty acids	Structure	Composition (wt %)
Palmitic acid	C16:0	45.9 ± 1.90
Stearic acid	C18:0	3.35 ± 0.41
Oleic acid	C18:1	38.1 ± 1.08
Linoleic acid	C18:2	9.1 ± 0.13
Others	-	3.6 ± 0.90

Table 2. Amounts of medium-chain-length polyhydroxyalkanoates (MCL-PHAs) produced by some *Pseudomonas* strains.

Strains	DCW (g/l)[a]	MCL-PHAs (% DCW)	Source[b]
P. fluorescens	0.65 ± 0.03	3.14 ± 0.06	KCTC 12453
P. chlororaphis	0.67 ± 0.08	5.02 ± 1.67	KCTC 12349
P. putida	1.41 ± 0.15	15.73 ± 2.17	KCTC 1452
P. aeruginosa	0.99 ± 0.07	3.94 ± 0.96	KCTC 1637
Pseudomonas sp.	0.43 ± 0.01	2.65 ± 0.33	KCTC 1640
P. gessardii	0.65 ± 0.11	5.13 ± 0.93	DSMZ 17152
P. moorei	0.90 ± 0.08	20.19 ± 4.87	DSMZ 12647
P. mohnii	1.05 ± 0.31	21.55 ± 4.53	DSMZ 18327
P. putida KT2440	1.16 ± 0.20	20.80 ± 2.73	Victor de Lorenzo (Spain)
P. putida S12	1.01 ± 0.16	24.87 ± 3.90	Victor de Lorenzo (Spain)

[a]DCW, dry cell weight.

[b]KCTC, Korean Collection for Type Cultures; DSMZ, German Collection of Microorganisms and Cell Cultures.

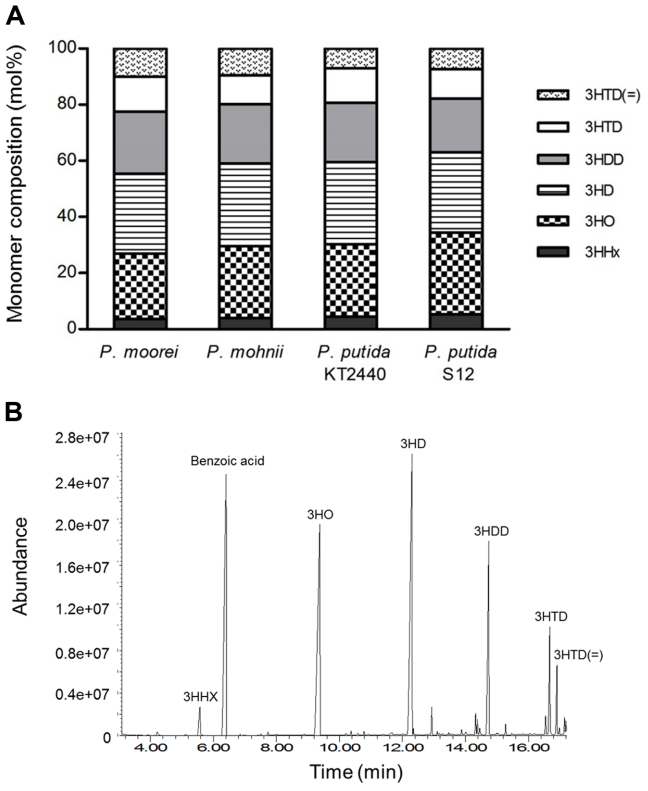

Fig. 1. Monomeric composition of medium-chain-length polyhydroxyalkanoates (PHAs) (mol%) produced by *Pseudomonas* strains during growth on sludge palm oil (**A**) and gas chromatography/mass spectrometry chromatogram of PHAs produced from *P. putida* S12 (**B**).

3-HHx, 3-hydroxyhexanoic acid; 3-HO, 3-hydroxyoctanoic acid; 3-HD, 3-hydroxydecanoic acid; 3-HDD, 3-hydroxydodecanoic acid; 3-HTD, 3-hydroxytetradecanoic acid; 3-HTD(=), 3-hydroxytetradecaenoic acid.

rate among the strains. In total, there were six types of monomers in the produced PHAs: 3-hydroxyhexanoic acid (3-HHx), 3-hydroxyoctanoic acid (3-HO), 3-hydroxydecanoic acid (3-HD), 3-hydroxydodecanoic acid (3-HDD), 3-hydroxytetradecanoic acid (3-HTD), and 3-hydroxy-tetradecaenoic acid (3-HTD(=)).

PHA properties are determined by monomer units, with PHA containing medium-carbon-chain monomers being more elastic, flexible, and sticky than PHA composed of short-carbon-chain monomers [23]. We expect that the PHAs produced by *Pseudomonas* from SPO are elastic because 3-HO (C8), 3-HD (C10), and 3-HDD (C12) are the major components. Although *P. putida* KT2440 has been previously reported to produce PHA, this is the first report of its use to produce PHA from SPO. In addition, this is the first report of PHA production by *P. moorei* and *P. mohnii* from fatty acids. Among the four best PHA producers, *P. putida* S12 exhibited the highest efficiency in producing MCL-PHA (Table 2). We therefore further assessed the characteristics of the MCL-PHA produced by *P. putida* S12 from SPO.

Fed-batch fermentation was conducted in a 5 L reactor (KoBioTech, Korea) with a working volume of 2 L. In brief, cells grown in 50 ml of LB medium were harvested and transferred to a fermenter containing E* medium and 10 g/l SPO. Growth conditions were maintained at 30°C and 200 rpm without pH control. After 24 h, 5 g/l SPO was fed to the cells, which were then cultured for an additional 24 h. The fermentation of *P. putida* S12 resulted in the extraction of 3.7 g/l PHA from 15 g/l SPO (yield of approximately 41%), and this PHA was then subjected to gel permeation chromatography and differential scanning calorimetry (DSC) analysis at the Korea Polymer Testing & Research Institute (KOPTRI, Korea) (Table 3). The weight average molecular weight (Mw) and number average molecular weight (Mn) of the PHAs were found to be approximately 106 kDa and 45 kDa, respectively, and the polydispersity index (PDI) was 2.33. The monodisperse products were found to have a PDI closer to 1 in other studies [1], indicating that the PHA produced by *P. putida* S12 has a broad molecular weight distribution. Diverse fatty acids in SPO were catabolized to various PHA precursors through the β-oxidation pathway, resulting in the production of different length of PHAs and distinctive characteristic PHA products. DSC analysis elucidated the thermal properties of the produced PHA; the glass transition temperature (Tg) was found to be –42°C and the melting temperature (Tm) was 35°C.

The results of this study demonstrate that low-quality SPO can be used as an environmentally friendly and inexpensive resource for the production of MCL-PHAs by *P. putida*. Moreover, the PHA produced from SPO exhibits

Table 3. Properties of medium-chain-length polyhydroxyalkanoates produced by *P. putida* S12 based on gel permeation chromatography and differential scanning calorimetry analysis.

Strain	Mn (Da)	Mw (Da)	PDI	Tg (°C)	Tm (°C)
P. putida S12	45,354	105,792	2.33	- 41.75	34.95

Mn, number average molecular weight; Mw, weight average molecular weight; PDI, polydispersity index; Tg, glass transition temperature; Tm, melting temperature.

properties that are attractive in a biopolymer. Although the production yield of PHAs produced from SPO using *Pseudomonas* was similar to that of PHAs produced from fatty acids in a previous study [24], and despite the promising results presented here, further improvement is needed for economical production of PHA. In the future, the production yield should be increased by metabolic flux engineering [25], utilization of other PHA producers such as *Cupriavidus necator*, *Rhodobacter sphaeroides*, and *Comamonas* sp. [26], and process development such as two-stage batch or high-cell-density fed-batch culture [27]. Production of PHAs from SPO may contribute to a low-cost method of bioplastic production and may create opportunities for new industrial applications of these eco-friendly products.

Acknowledgments

This work was supported by the New & Renewable Energy Core Technology Program of the Korea Institute of Energy Technology Evaluation and Planning (KETEP), funded by the Ministry of Trade, Industry & Energy, Republic of Korea; the Pioneer Research Center Program (NRF-2013M3C1A3064780); and the Intelligent Synthetic Biology Center of Global Frontier Project (NRF-2015M3A6A8065831), funded through the National Research Foundation of Korea by the Ministry of Science, ICT & Future Planning and the Research Initiative Program of KRIBB.

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