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Characterization of a Tacky Poly(3-Hydroxyalkanoate) Produced by *Pseudomonas chlororaphis* HS21 from Palm Kernel Oil

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Abstract *Pseudomonas chlororaphis* HS21 was isolated from a soil sample and found to produce medium-chain-length polyhydroxyalkanoates (MCL-PHAs) using palm kernel oil (PKO) as the sole carbon source. Up to 3.3 g/l dry cell weight containing 45% MCL-PHA was produced, when the strain was grown for 21 h in a jar fermentor culture containing 5 g/l PKO. The polymer produced from PKO consisted of unsaturated monomers of 7.3% 3-hydroxy-5-*cis*-tetradecenoate and 2.3% 3-hydroxy-5,8,-*cis,cis*-tetradecadienoate as well as saturated even-carbon number monomers ranging from C_6 to C_{14}, as determined by GC and EI GC/MS. The PHA was a transparent, sticky material at room temperature. A differential scanning calorimetric analysis revealed that the polymer was amorphous with a –44°C glass transition temperature. The number average molecular weight and polydispersity index of the PHA were 83,000 and 1.53, respectively. Although the PHA was practically biodegradable, its degradability was lower than that of poly(3-hydroxyoctanoate) based on a comparison of the clear zones formed by growing PHA depolymerase-producing bacteria on an agar plate containing the respective polymers.

Key words: Medium-chain-length poly(3-hydroxyalkanoate), MCL-PHA, palm kernel oil, *Pseudomonas chlororaphis* HS21

Poly-(*R*)-hydroxyalkanoates (PHAs) are an energy reserve material accumulated intracellularly by numerous bacteria, and have been drawing a great deal of attention as candidates for biodegradable plastic or elastomer [27]. These polyesters can be primarily divided into two classes of short-chain-length (SCL) PHAs and medium-chain-length (MCL) PHAs, according to the carbon-chain-length of the constituents: SCL-PHAs consist of (*R*)-hydroxyalkanoates of C_3-C_5, while MCL-PHAs are comprised of (*R*)-hydroxyalkanoates of C_6-C_{14} [19]. SCL-PHAs are thermoplastics with a high degree of crystallinity, whereas most MCL-PHAs are elastomers or adhesives with a low degree of crystallinity and low melting temperature. Up to now, more than 150 different hydroxyalkanoates have been detected as constituents in bacterial PHAs [25, 27].

It has already been reported that *Pseudomonas oleovorans* and *P. putida*, representative MCL-PHA-producing microorganisms, can produce various aliphatic and aromatic MCL-PHAs, when they are grown with free fatty acids with corresponding chemical structures [10–12, 14, 17, 18, 22, 23]. However, recent studies on PHA biosynthesis have demonstrated that several *Pseudomonas* spp. can also produce significantly large amounts of MCL-PHAs from renewable resources such as plant oils and tallow, which are inexpensive carbon substrates [1, 3, 6, 21, 24]. Among these polymers, MCL-PHAs with a high fraction of unsaturated repeating units are of particular interest, because they possess the material property of adhesives. However, the unsaturated groups in the side chain can also be used as reaction centers for the preparation of polymers with different properties through chemical modifications [2, 16].

Recently, due to their excellent biodegradability and biocompatibility, PHAs have attracted significant industrial attention and are expected to be useful biomaterials capable of replacing synthetic polymers, including petroleum-based plastics, particularly in biomedical fields. However, their high production cost remains one of the critical problems that need to be solved to extend the use of PHAs as commercial biomaterials. Therefore, increasing the polymer yield with a less expensive substrate would make PHA production more economical.

Accordingly, the current study was undertaken to isolate a bacterial strain, HS21, capable of efficiently producing a

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tacky MCL-PHA from palm kernel oil (PKO), along with some of the material properties of the polymer synthesized by this isolate.

MATERIALS AND METHODS

Palm Kernel Oil

Commercial PKO, an extract from the nut of the oil palm (*Elaeis guineensis Jacq.*) fruit, was purchased from Malaysia and consisted of 44% lauric ($C_{12:0}$), 14% myristic ($C_{14:0}$), and 17% oleic acids ($C_{18:1}^{\Delta 9}$) as the major fatty acids, and octanoic ($C_{8:0}$), decanoic ($C_{10:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), and linoleic acids ($C_{18:2}^{\Delta 9,12}$) as the minor components [4].

Culture of Bacterium and Preparation of PHAs

The isolate, designated HS21, was isolated from a soil sample by enrichment using a mineral salt medium [11] containing 5 g/l of PKO. Shake flask cultures were carried out aerobically in a 500-ml Erlenmeyer flask containing 100 ml of a mineral salt medium. Batch fermentation was conducted in a 5-l jar fermentor (Korea Fermentor Co. Ltd.) with a working volume of 3 l. The mineral salt medium supplemented with 5 g/l of PKO was inoculated with a 10% (v/v) inoculum that had been cultivated for 30 h in the same medium, except that it contained 3 g/l of PKO as the sole carbon source. The temperature and pH were automatically controlled at optimal values of 30°C and 7.0, respectively. The airflow rate was 0.65 vvm and the agitation speed 250 rpm. The cell growth was monitored spectrophotometrically at 660 nm. The cell cultivation was stopped approximately 2 h after the growth reached the stationary phase. The cells were then harvested by centrifugation, followed by lyophilization. The PHA was isolated from the lyophilized cells by extraction with hot chloroform using a Soxhlet apparatus [5]. The extracted crude PHA was then purified by repeated precipitation into vigorously stirred cold methanol.

Analytical Methods

The relative concentrations of the synthesized PHA monomeric units were determined by a gas chromatography (GC) analysis, as previously described [11]. The identification of the PHA monomeric units was carried out by electron impact gas chromatography/mass spectrometry (EI GC/MS) and ^{1}H-NMR spectroscopy. The EI GC/MS analysis was conducted using a Hewlett-Packard 5988 GC/MS system (Avondale, U.S.A.), while the NMR spectroscopy was performed using a Bruker 500 NMR spectrometer (Rheinstetten, Germany) [17]. The differential scanning calorimetry (DSC) analysis was carried out using a Perkin-Elmer DSC 7 (Foster, U.S.A.). The molecular weights of the PHAs were determined using a gel permeation chromatography system equipped with a Waters 6000 solvent delivery system, RI detector, and Rheodyne injector (Miliford, U.S.A.) [10].

Analysis of Residual NH_4^+ and PKO in Medium

Approximately 50 ml of the culture was taken at different growth times and centrifuged. The supernatant (5 ml) of the culture was also taken, and 2 ml of chloroform solution containing 0.3 wt% benzoic acid as an internal standard was added. The mixture was acidified with 50 µl of 10 N HCl, then agitated vigorously. The organic layer was taken for analysis, and 1 µl aliquot of the chloroform solution was injected into a gas chromatograph. The amount of residual PKO in the medium was calculated from the area of the methyl laurate peak in the gas chromatogram using the methyl benzoate peak as the internal standard. The concentration of NH_4^+ in the medium was determined using Nessler's reagent [7].

Nucleotide Sequence Accession Number

The 16S rDNA sequence of strain HS21 has been deposited in GenBank under accession no. AF509334.

RESULTS AND DISCUSSION

Identification of Isolate

Isolate HS21 was found to be a Gram-negative rod, motile, and non-spore-forming bacterium. The cellular fatty acids consisted of 10:0 3OH (4.5%), 12:0 2OH (6.4%), 12:0 3OH (5.0%), 16:1 w7c/15 iso 2OH (22.2%), 16:0 (35.9%), 17:0 CYCLO (14.3%), and 18:1 w7c (11.7%). A phylogenetic analysis of the nucleotide sequences of the 16S rDNA showed that the isolate was clearly grouped with *Pseudomonas* species, with the highest similarity of 98.6% to *Pseudomonas chlororaphis* LMG 5004^T. Based on these results, the isolated strain HS21 was identified as *P. chlororaphis*.

Biosynthetic Pattern of MCL-PHAs of *P. chlororaphis* HS21

Table 1 shows the composition of the PHAs biosynthesized by *P. chlororaphis* HS21 from hexanoic to dodecanoic acids. The organism produced homopolyesters of poly(3-hydroxyhexanoate) and poly(3-hydroxyheptanoate), when it was cultivated on a medium containing hexanoate and heptanoate, respectively. The result was comparable to other well-studied *Pseudomonas* species, such as *P. stutzeri* [28], *P. putida* [13], and *Pseudomonas* sp. DSY-82 [9], where the PHAs produced from hexanoate generally contained 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate units, and the PHAs synthesized from heptanoate composed of 3-hydroxyheptanoate and 3-hydroxynonanoate. In contrast, *P. chlororaphis* HS21 also synthesized copolyesters consisting of either 3-

Table 1. Compositions of PHAs produced from alkanoic acids of C_6-C_{12} and PKO.

PHA isolate	3-Hydroxymethyl esters[a] (%)												
	C_6	$C_{7:1}$	C_7	C_8	$C_{9:1}$	C_9	C_{10}	$C_{11:1}$	C_{11}	C_{12}	C_{14}	$C_{14:1}$	$C_{14:2}$
PHA-C_6	100												
PHA-C_7			100										
PHA-C_8	7.5			92.5									
PHA-C_9			24.6			75.4							
PHA-C_{10}	7.0			61.3			31.7						
PHA-C_{11}			21.9			60.5			17.6				
PHA-$C_{11:1}$		16.1			65.8			18.1					
PHA-C_{12}	7.5			57.0			24.7			10.8			
PHA-PKO	4.7			34.7			32.5			1.4	4.0	7.3	2.3

Cells were cultivated in a 500-ml Erlenmeyer flask containing 100 ml of a mineral salt medium supplemented with alkanoic acids or in a 5-l jar fermentor containing 3 l of the same medium supplemented with PKO (details in Materials and Methods).

[a]GC area%. The monomer structures were determined by EI GC/MS and ^{1}H-NMR spectroscopy analyses.

hydroxyoctanoate (3HO) or 3-hydroxynonanoate as the major constituent from octanoic to dodecanoic acids.

It is well-known that the PHA biosynthesis in bacteria is closely linked to three different metabolic routes [27]: (i) *de novo* fatty acid biosynthesis pathway that produces (*R*)-3-hydroxyacyl-CoA precursors from nonrelated carbon sources such as glucose and gluconate; (ii) fatty acid degradation by β-oxidation; (iii) chain elongation reaction, in which acyl-CoA is extended with acetyl-CoA. In this study, *P. chlororaphis* HS21 did not utilize glucose or gluconate as the sole carbon source for PHA production (data not shown). However, the PHAs synthesized by the organism from alkanoic acids only contained 3-hydroxyalkanoates derived from the precursors produced by the β-oxidation cycle of the given alkanoic acids (Table 1). Therefore, these results indicate that the PHA biosynthetic pathway of *P. chlororaphis* HS21 is closely linked to the β-oxidation cycle of fatty acids, yet not related to *de novo* fatty acid biosynthesis and chain elongation reactions. A similar trend with the PHA biosynthetic pattern of *P. chlororaphis* HS21 was also observed in *P. oleovorans* [18]. Nevertheless, the majority of MCL-PHA-producing *Pseudomonas* strains have been reported to exhibit either *de novo* fatty acid biosynthesis or a chain elongation reaction as an additional metabolic route for the formation of monomer precursors from polymer-producing substrates [8, 9, 13, 26, 28].

Production of Tacky MCL-PHA from PKO

To examine the effect of PKO concentration on cell growth and PHA production, batch cultures were carried out with varying amounts of PKO ranging from 3 to 30 g/l. The maximum biomass yield was relatively high (0.62–0.67 g/g) when the initial PKO concentration was between 5 and 10 g/l, and was significantly decreased when PKO concentration exceeded 20 g/l (data not shown). Although PKO concentration seemed to affect cell growth, PHA content in all cases did not vary significantly and amounted to 35–48% of dry cell weight. A typical time course for growth and PHA accumulation of *P. chlororaphis* HS21 in batch culture is shown in Fig. 1. The cell growth and PHA accumulation of this organism occurred rapidly due to the simultaneous utilization of PKO and $(NH_4)_2HPO_4$ throughout the cultivation period. In this case, most of the PKO in the culture medium was depleted after 17 h of cultivation and a dry cell weight (DCW) of 3.3 g/l was achieved together with a PHA of 1.49 g/l (45% of DCW) at the end of the 21 h experiment. These results demonstrate that *P. chlororaphis* HS21 was capable of efficiently utilizing PKO for growth and PHA production. Tan *et al.* [24] previously reported on the production of a MCL-PHA by *P. putida* PGA1 using 5 g/l of saponified PKO. However, in that case, the amount of cells obtained and PHA content were only 3.0 g/l and 37 wt%, respectively, which was less than that obtained in the current study. Moreover, due to the absence of lipase activity in *P. putida* PGA1, the PKO had to be saponified for use as a sole carbon source for cell growth. From these descriptions, it is suggested that the direct production of MCL-PHA from PKO is more cost

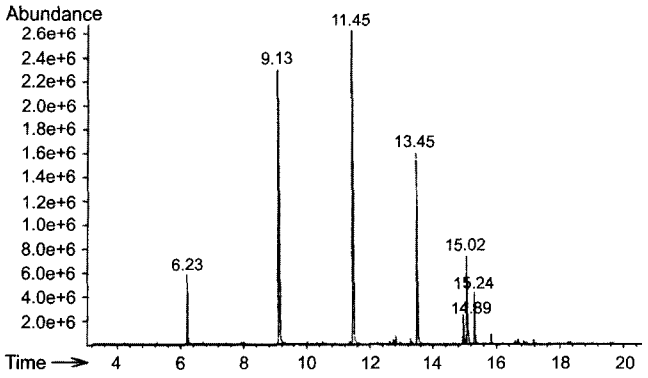

Fig. 1. Gas chromatogram of methanolyzed samples of PHA produced from PKO.

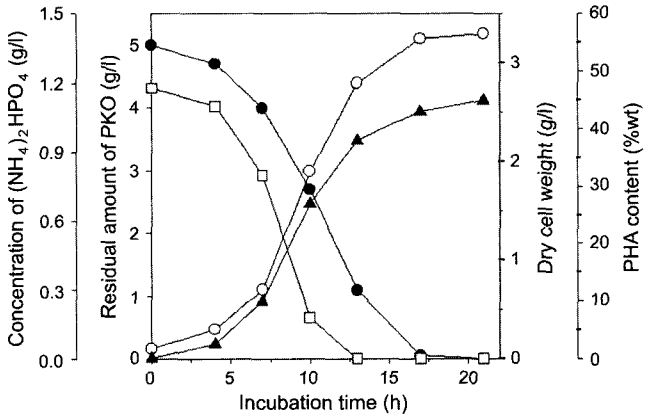

Fig. 2. Time course of cell growth and PHA accumulation during the batch fermentation of *P. chlororaphis* HS21 in a mineral salt medium containing 5 g/l of PKO as the sole carbon source.
Symbols: (○) dry cell weight; (▲) PHA content; (□) residual amount of $(NH_4)_2HPO_4$; (●) residual amount of PKO.

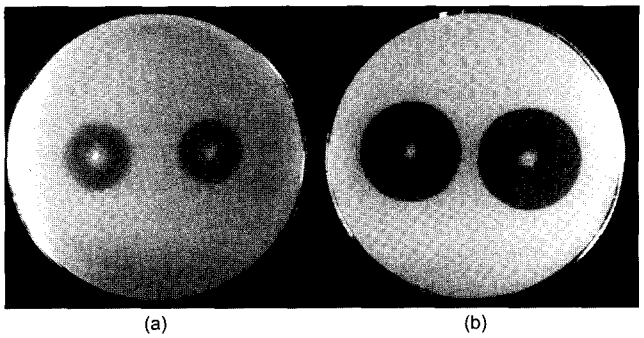

Fig. 3. Biodegradation of PHA_{PKO} (a) and PHO (b) by *P. alcaligenes* LB19 (left colony) and *Pseudomonas* sp. RY-1 (right colony) grown on mineral salt agar medium containing respective polymers (0.1% wt/v) for 4 days at 30°C.

effective and can be achieved using *P. chlororaphis* HS21 with a high lipase activity.

Characterization of PHA Produced from PKO

Figure 2 shows that the PHA produced from PKO (PHA_{PKO}) consisted of seven repeating units with different retention times. The EI mass spectra of the different peaks in the gas chromatogram revealed that all the peaks contained ion fragments with *m/z* values of 43, 71, and 103, which are characteristic ones produced from methyl 3HAs (Table 2). The monomer structures of the PHA_{PKO} were also identified by an ^{1}H-NMR spectroscopy analysis (data not shown). The results of the composition analysis of the PHA_{PKO} copolyester are listed in Table 1. When grown with PKO, *P. chlororaphis* HS21 preferentially incorporated similar amounts of 3HO and 3-hydroxydecanoate units as the main constituents in the PHA_{PKO}. This result was significantly different from that of Tan *et al.* [24], who demonstrated that the PHA produced by *P. putida* PGA1 from saponified PKO was a copolyester containing 82.9% 3HO as the major monomer. In addition, as shown in Table 1, the PHA_{PKO} produced by *P. chlororaphis* HS21 contained unsaturated repeating units of 7.3% 3-hydroxy-5-*cis*-tetradecenoate and 2.3% 3-hydroxy-5,8,-*cis,cis*-tetradecadienoate, while it was previously reported that the PHA synthesized by *P. putida* PGA1 only contained 1.3% unsaturated units [24].

The PHA_{PKO} was a transparent, sticky material at room temperature, similar to the PHAs synthesized from either 10-undecenoate or its mixtures with nonanoate [11], while the PHA synthesized from saponified PKO by *P. putida* PGA1 is an elastomer [24]. The stickiness may have been related to the surface free energy and surface roughness of the polymer [20]. A DSC analysis indicated that the PHA_{PKO} was a noncrystalline polymer, possessing a −44°C glass transition temperature without a melting temperature. The number average molecular weight and polydispersity index of the PHA were 83,000 and 1.53, respectively, as determined by GPC. The molecular mass distribution of the PHA_{PKO} was comparable to that of other polymers produced by *P. putida* [24], *P. saccharophila* [21], and *P. resinovorans* [3] from various vegetative oils, as the polydispersity indices of the PHAs produced by the above microorganisms are generally more than 2.0.

The PHA_{PKO} was practically biodegradable, however; even though the polymer was completely amorphous, its degradability was always approximately two times lower than that of poly(3-hydroxyoctanoate), as determined by growing MCL-PHA depolymerase-producing microorganisms

Table 2. EI GC/MS analysis of methanolyzed samples of PHA synthesized by *P. chlororaphis* HS21 from PKO.

Retention time (min)	m/z	Methyl ester of constituent
6.231	43, 55, 61, 71, 74, 97, 103, 117, 128, 145	methyl ester of 3-hydroxyhexanoate ($C_{6:0}$)
9.093	43, 55, 61, 71, 74, 83, 103, 125, 156, 173	methyl ester of 3-hydroxyoctanoate ($C_{8:0}$)
11.440	41, 43, 55, 61, 71, 74, 103, 127, 152, 184, 201	methyl ester of 3-hydroxydecanoate ($C_{10:0}$)
13.451	41, 43, 55, 61, 71, 73, 83, 103, 138, 180, 229	methyl ester of 3-hydroxydodecanoate ($C_{12:0}$)
14.886	43, 55, 61, 71, 79, 91, 103, 105, 119, 133, 152, 162, 193, 236	methyl ester of 3-hydroxytetradecadienoate ($C_{14:2}$)
15.024	43, 55, 61, 71, 84, 103, 121, 126, 135, 150, 164, 206, 225, 238	methyl ester of 3-hydroxytetradecenoate ($C_{14:1}$)
15.247	41, 43, 55, 61, 71, 74, 97, 103, 128, 137, 166, 183, 191, 208	methyl ester of 3-hydroxytetradecanoate ($C_{14:0}$)

(*Pseudomonas* sp. RY-1 [15] and *P. alcaligenes* LB19 [13]) on an agar plate containing the respective polymers (Fig. 3). As such, the low degradability of the PHA_{PKO} may have been due to its irregular microstructure protecting the ester bonds from enzyme attack.

CONCLUSION

The isolate *P. chlororaphis* HS21 produced MCL-PHAs from various free fatty acids and PKO via the β-oxidation pathway of fatty acids as the sole PHA biosynthetic route. The current results indicate that *P. chlororaphis* HS21 is capable of producing a high yield of MCL-PHA from PKO itself, without the need for saponification. Accordingly, the economical production of MCL-PHAs from renewable raw materials, such as PKO or other plant oils, is possible using this organism. A further study on the development of a new fermentation strategy for the economical production of MCL-PHAs is currently underway.

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