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NOTE

Isolation of an Aromatic Polyhydroxyalkanoates-degrading Bacterium

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Abstract Five microorganisms capable of degrading an aromatic medium-chain-length polyhydroxyalkanoate (PHA_{MCL}), poly(3-hydroxy-5-phenylvalerate) (PHPV), were isolated from wastewater-treatment sludge. Among the isolates, JS02 showed degrading activity consistantly during several transfers. The isolate JS02 could hydrolyze another aromatic MCL copolyester, poly(3-hydroxy-5-phenoxyvalerate-*co*-3-hydroxy-7-phenoxyheptanoate), [P(5POHV-*co*-7POHH)], and other short-chain-length PHAs (PHA_{SCL}) such as poly(3-hydroxybutyrate) [P3(HB)], poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)], and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] with relatively low activity. The culture supernatant of JS02 showed hydrolyzing activity for the *p*-nitrophenyl esters of fatty acids.

Key words: Isolation of bacteria, degradation, aromatic medium-chain-length polyhydroxyalkanoate (PHA_{MCL}), poly (3-hydroxy-5-phenylvalerate) (PHPV), PHA depolymerase

Polyhydroxyalkanoates (PHAs) are synthesized and accumulated in many bacteria [1, 2, 12]. These microbial polyesters are deposited intracellularly as granular inclusion when nutrients other than carbon sources become limiting, thus act as a reservoir of carbon and/or energy. These bacterial polyesters have attracted industrial and agricultural attention due to their complete biodegradability to CO_2 and H_2O. Since the discovery of poly(3-hydroxybutyrate) [P(3HB)] in 1926, much work has been focused on the short-chain-length PHA (PHA_{SCL}) [1, 2]. Recently, with the discovery of monomers other than 3-hydroxybutyrate as constituents of PHA, numerous PHA-degrading bacteria have been isolated and characterized. There have been many reports concerning the synthesis of aliphatic medium-chain-length PHAs (PHA_{MCL}) with 6 to 14 carbon atoms [3, 4, 8, 9, 12, 14, 15], but only four of them described the degradation of aliphatic PHA_{MCL} [3, 4, 8, 9]. The poly(3-hydroxy-octanoic acid) [P(3HO)] depolymerase of *Pseudomonas fluorescens* GK13 is the only PHA_{MCL} depolymerase that has been purified and characterized [9]. The possibility of phenyl group incorporation into R side-chains of the microbial polyester has been studied [7], however, little has been reported for the synthesis of aromatic PHA_{MCL} [5, 10, 13, 16]. In this study, we describe the isolation of bacteria capable of degrading an aromatic PHA_{MCL}, poly(3-hydroxy-5-phenylvalerate) (PHPV), and hydrolyzing specificities of the most active strain, JS02, for other PHAs and artificial fatty acid esters. To the best of our knowledge, this is the first report of aromatic PHA_{MCL}-degradation.

The homopolymer of PHPV was produced in *Pseudomonas putida* BM01 [16] with a modification of cosubstrate concentration (Song, J. J. and S. C. Yoon. 1998. unpublished data). The cells were grown in a mineral medium containing 30 mM phenylvalerate and 20 mM butyrate as carbon sources for 40 h at 30°C. The mineral medium used in this study was a modification of Song *et al.* [11] and Yoon *et al.* [16]. The composition of the mineral medium was as follows: 8 mM $(NH_4)_2SO_4$, 30 mM KH_2PO_4/Na_2HPO_4, 1 mM $MgSO_4 \cdot 7H_2O$, and 2 ml of a microelement solution per liter of distilled water. The microelement solution contained 0.029% $ZnSO_4 \cdot 7H_2O$, 0.278% $FeSO_4 \cdot 7H_2O$, 0.198% $MnCl_2 \cdot 4H_2O$, 0.03% H_3BO_3, 0.017% $CuCl_2 \cdot 2H_2O$, 0.281% $CoSO_4 \cdot 7H_2O$, 0.167% $CaCl_2 \cdot 2H_2O$, and 0.002% $NiCl_2 \cdot 7H_2O$ per liter of distilled water. To obtain the polymer, the harvested cells were washed with acetone, lyophilized, and refluxed with chloroform in a Soxhlet apparatus; subsequently, the chloroform solution was concentrated tenfold by evaporation and precipitated with ten volumes of ethanol, dried at room temperature, and dissolved in chloroform at a final concentration of 2.5% (w/v), as described by Timm and Steinbüchel [15]. The purity of the PHPV homopolymer produced by *P. putida* BM01 was estimated to be 99.1% by gas chromatographic analysis (Fig. 1).

To isolate PHPV-degrading microorganisms, samples were collected from wastewater, rivers, landfills, and

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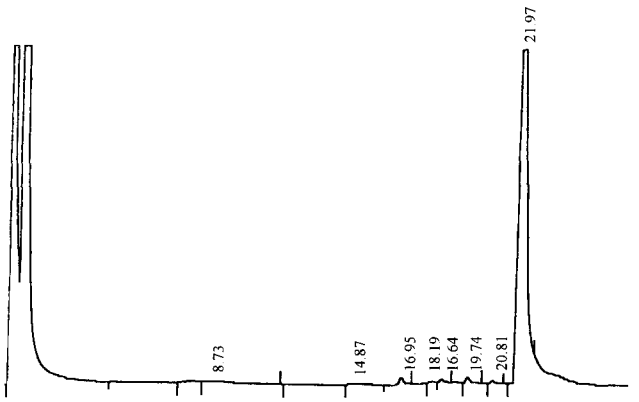

Fig. 1. A gas chromatogram showing the purity of the PHPV homopolymer produced by *P. putida* BM01.

The cells were grown in a mineral medium containing 30 mM phenylvalerate and 20 mM butyrate as carbon sources for 40 h at 30°C. The purity was analyzed with a Hewlett-Packard HP5890A gas chromatograph equipped with an OV25 column as described previously [10]. The largest major peak corresponds to the PHPV monomer.

wastewater-treatment plants near Sunchon, suspended in sterilized water, and spread on nutrient agar (NA) plates overlaid with 0.3% PHPV. PHPV agar plates were made as follows: seven volumes of cold distilled water were added to 2.5% (w/v) PHPV solution in chloroform, mixed with a blender, and then the chloroform was evaporated. The resultant solution was dispersed in NA, autoclaved and overlaid onto the top of NA plates. From the wastewater-treatment sludge, five microorganisms capable of degrading PHPV were isolated (Fig. 2). Among them, isolate JS02 showed degrading activity consistantly during several transfers (data not shown). The isolate JS02 grew better in Luria-Bertani (LB) medium than in nutrient media, but hydrolyzed PHPV better on nutrient agar (NA) than on LB agar medium

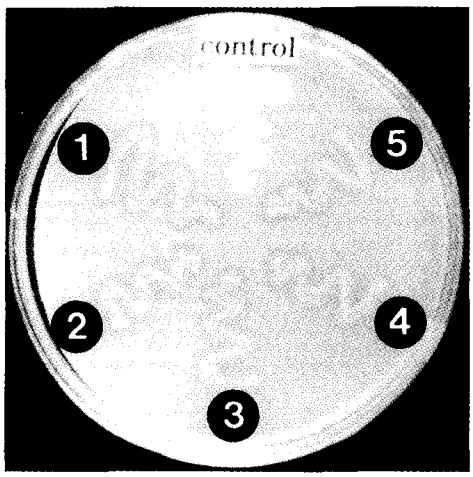

Fig. 2. PHPV depolymerase activities of the isolates on an agar plate overlaid with 0.3% PHPV.

Symbols: ①, isolate JS01; ②, JS02; ③, JS03; ④, JS04; ⑤, JS21.

Table 1. Growth and PHPV-degrading activity of the isolate JS02 grown in different media*.

Media		Culture time (h)			
		12	16	22	30
1/4×NA	Growth	+	+	+	+
	Activity	-	-	+	+
1×NA	Growth	++	++	+++	+++
	Activity	-	+	++	+++
1×LB	Growth	+++	+++	+++	+++
	Activity	-	-	△	△

*Isolate JS02 was grown on agar plates overlaid with PHPV. The PHPV-degrading activity was determined by the relative halo sizes around the colonies: -, no zone; △, incomplete zone; +, small clear zone; ++, medium clear zone; +++, large clear zone. Growth was separately determined by measuring A_{600} in broth media: +, <0.5; ++, <1.0; +++, ≥1.0.

(Table 1). No significant increase in degrading activity was observed when the isolate JS02 was grown in NB in the presence of 0.15% PHPV (data not shown). This might indicate non-inducible nature of the enzyme by the substrate.

The isolate JS02 could degrade another aromatic MCL copolyester, poly(3-hydroxy-5-phenoxyvalerate-*co*-3-hydroxy-7-phenoxyheptanoate) [P(5POHV-*co*-28% 7POHH)] with less activity, about one-third of the PHPV degrading activity. The copolyester [P(5POHV-*co*-28% 7POHH)] was synthesized by *P. putida* BM01 in the presence of 20 mM of 11-phenoxyundecanoate (11-POU) as described by Song and Yoon [10] (Table 2). The isolate JS02 was also able to hydrolyze on solid media a PHA_{SCL} polyester, poly(3-hydroxybutyrate) [P(3HB)], and PHA_{SCL} copolyesters such as poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-25% 4HB)], and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-45% 3HV)] produced by *Alcaligenes eutrophus* [6] (Table 2). To date, eight bacteria showing the PHA_{MCL} depolymerase activity have been reported, all in the last 5 years; five *Pseudomonas* species and one *Xanthomonas maltophilia* strain [8], *P. maculicola* [3], and *P. fluorescens* GK13 [6]. Strain JS02 isolated in this study seemed to be similar to *Xanthomonas maltophilia* strain [8] in that it could degrade both PHA_{SCL} and PHA_{MCL}. Even though

Table 2. Degradation of PHAs by the culture supernatant of the isolate JS02 on agar plates overlaid with PHAs.

PHAs	Hydrolyzing activity
PHPV	+++
P(3HB)	+
P(3HB-*co*-25% 4HB)	++
P(3HB-*co*-45% 3HV)	++
P(5POHV-*co*-28% 7POHH)	+

The PHPV-degrading activity was determined by the halo size around the colony. +, small clear zone; ++, medium clear zone; +++, large clear zone.

Table 3. Degradation of PNP-esters of fatty acids by the culture supernatant of JS02.

Substrate	Hydrolyzing activity (U/ml)	Relative activity (%)
PNP-butyrate	0.011	55.0
PNP-hexanoate	0.020	100.0
PNP-octanoate	0.013	65.0
PNP-decanoate	0.005	25.0
PNP-dodecanoate	0.002	10.0
PNP-tetradecanoate	0.00028	1.4
PNP-hexadecanoate	0.00038	1.9

The supernatant was added into a reaction mixture prewarmed at 30°C. The reaction mixture contained 10 μl of 10 mM PNP-esters in water or ethanol, 50 to 100 μl of the supernatant, and 50 mM Tris-HCl buffer (pH 9.0) to a total volume of 1 ml. One unit was defined as the amount of enzyme producing 1 μmol of *p*-nitrophenol in 1 min under the above condition. The amount of *p*-nitrophenol was determined by the absorbance at 400 nm.

the lipases from *P. alcaligenes* and *P. aeruginosa* showed PHA_{MCL} depolymerase activity, the activity was very weak [4]. The supernatant of JS02 showed hydrolyzing activity for the *p*-nitrophenyl (PNP) esters of fatty acids such as PNP-hexanoate, PNP-octanoate, PNP-butyrate, and PNP-decanoate, in decreasing order (Table 3). Isolate JS02 is gram-negative, straight rod, 1.5~2.0 μm in length, motile, and catalase positive, and forms yellow colonies on agar media. The exact affiliation of the isolate is under investigation.

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