Isolation of a microorganism capable of degrading poly-(L-lactide)

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The isolation of poly-(L-lactide) (PLA)-degrading microorganisms was investigated. A PLA-degrading actinomycete, strain No. 3118, was isolated and tentatively identified as a member of the genus *Amycolatopsis*. The optimum conditions for degradation of PLA were 43°C at about pH 7 in a mineral salt medium with a low concentration of organic nutrients (0.002% yeast extract). The original shape of PLA film ($M_w=2.3 \times 10^5$ after sterilization, 20 μm thick) disappeared within 2 weeks. Lactic acid was detected after the film was incubated with culture supernatant.

Key Words—*Amycolatopsis*; poly-(L-lactide) degradation; poly-(L-lactide)-degrading bacteria; poly-(L-lactide)-degrading conditions

Plastics are beneficial because of their facility, lightness, ease in processing, transparency, and low cost. Although some plastics are reused and recycled, the disposal of a large quantity of used plastic waste poses a great problem. Spacious land is required for burying waste material, and poisonous gas is often emitted when plastic waste is incinerated. Furthermore, abiotic plastics spoil the scenery and the ecological systems of many forms of life. Considering these problems, biodegradable plastics have been the subject of many research papers. Several kinds have been produced, such as poly (β -hydroxybutyrate), polycaprolactone, and polyglycolic acid, and depolymerases capable of degrading these materials have been reported (Benedict et al., 1983; Chu, 1981; Kemnitzer et al., 1992; Reeve et al., 1994; Tanino et al., 1982). Poly-(L-lactide) (PLA), one of these biodegradable plastics, however, is expected to be used widely since its cost has decreased recently; already it is used in the medical field (Leenslag et al., 1987; Penning et al., 1993). PLA and other lactic acid-containing polymers are synthesized chemically (Dubois et al., 1991; Kricheldorf et al., 1992; Penning et al., 1993). Apart from its biodegradability, PLA has attracted attention as a material that can be made from renewable resources such as starch because lactic acid is produced through the fermentation of starch or glucose. It is completely harmless compared with abiotic polymers when used in vessels for edible products. PLA is known to degrade slowly in natural soil, but it

takes a long time for degradation to start. It can be hydrolyzed enzymatically (Ebeling et al., 1974; Fukuzaki et al., 1989; Reeve et al., 1994). Nevertheless, only a few reports have been made so far on the direct degradation of PLA by microorganisms.

One report concerns a mold capable of assimilating soluble PLA-oligomer and PLA-copolymer with glycerol (Torres et al., 1996). Furthermore, Pranamuda et al. (1997) reported the degradation of PLA by *Amycolatopsis* sp.

In the present study, we isolated a microorganism capable of efficiently degrading homo-PLA film in liquid culture. This strain differs from the one reported by Pranamuda et al. (1997), especially in terms of growth temperature and spore formation. In this report, we describe the identification of the PLA-degrading organism, the PLA-degradation conditions, and the products found in the culture supernatant.

Materials and Methods

Medium. A basal medium with the following composition was used (per liter): K_2HPO_4 , 1.55 g; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.85 g; $(\text{NH}_4)_2\text{SO}_4$, 2.0 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1 mg; yeast extract, 20 mg; agar (solid medium), 15 g.

PLA film preparation. PLA ($M_w=3.2 \times 10^5$) from Shimadzu Co., Ltd. (Kyoto, Japan) consisting of 99.8% L-lactic acid was used. PLA granules were dis-

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solved in chloroform at 2.0% (wt/vol). The solution was poured into Petri dishes and dried. PLA film 20 μm thick was prepared. Molecular weight after sterilization was 2.3×10^5 , and no oligomer was found. These molecular weights were measured with GPC (Shimadzu Co., Ltd.).

Isolation. Soil supernatant, pond water or river water, was spread onto solid medium, and the plates were overlaid with PLA film. Colonies growing and appearing on the film were cultured in 10 ml of liquid medium containing about 0.2% wt/vol PLA film at 30°C for 8 weeks. The strains that decreased the film weight by more than 5.5% were cultured again in the liquid medium at 30°C and 37°C for 4 weeks, and strains that efficiently decreased the film weight were isolated. Highly efficient PLA-degrading strains were obtained by repeated adaptation and selection procedures.

Identification of the strain. Czapek medium was used to grow the isolated microorganisms. Peptidoglycan type, mycolic acid, menaquinone, fatty acid pattern, and 16S rDNA sequence were analyzed by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany). Other characteristics were investigated by the authors. The strain was classified according to DSMZ and Bergey's Manual of Determinative Bacteriology (Halt et al., 1993).

Measurement of film weight after cultivation. PLA film was taken out from the culture tubes, washed thoroughly with distilled water, and dried up overnight in a desiccator. All visible pieces of film were recovered.

Analyses of the products. Enzyme reaction with supernatant was carried out aseptically. Film was wiped with 70% ethyl alcohol and irradiated with UV light. Test tubes with sealed caps were autoclaved, and concentrated supernatant was filtrated with membrane filter (0.22 μm pore size, Millipore Co., Bedford, MA, USA). Formed lactic acid was measured with lactate dehydrogenase (Stolzenbach, 1966). Thin-layer chromatography was developed with a solvent system of the upper solution of *n*-butyl alcohol, acetic acid, and water (4:1:5). Bromocresol Green was used as a colored indicator.

Results and Discussion

Isolation and identification

About 300 strains from 50 samples of soil, pond water and river water were isolated on plate culture and cultured in the liquid medium. After 8 weeks at 30°C under static conditions, 2 strains that decreased film weight by more than 50% were isolated. This shows that only a few microorganisms were capable of growth in liquid medium containing PLA film, al-

though PLA-degrading microorganisms were often found on plate culture. Because these two isolates were found to be identical, only the results for strain No. 3118 are presented in Table 1. This strain showed the typical form of an actinomycete after 1 day of cultivation on a plate of Czapek medium, dividing into rods after 2 days of cultivation. The characteristics of strain No. 3118 were found to be consistent with those of members of the genus *Amycolatopsis*. The sequence of the first 500 bp of 16S rDNA showed 96.9% similarity to that of *A. mediterranei*, indicating that strain No. 3118 is a new species of the genus *Amycolatopsis* because a similarity value of more than 99.4% is required to identify the species. Therefore we tentatively identified No. 3118 as a member of genus *Amycolatopsis*. This strain differs from the strain reported by Pranamuda et al. (1997) in the following characteristics. It was found to have spores and spore chains. This strain could grow at a temperature up to 48°C, it could grow in the presence of 7% NaCl, and L-rhamnose is utilized. Because 16S rDNA sequence similarity was not described in the case of an *Amycolatopsis* reported by Pranamuda et al. (1997), we could not compare our strain with theirs (Pranamuda et al., 1997). However, it is clear that No. 3118 is different from the strain isolated by Pranamuda et al. (1997).

Effects of temperature and nutrients on degradation of PLA

The marked degradation of PLA on incubation at 43°C was seen at about pH 7. In the practical use of this microorganism, conditions such as temperature and nutrient content of the medium will be important factors affecting the degradation. Yeast extract was employed as a nutrient. Because the strain grew within a wide range of temperatures (Table 1), degradation of PLA at various temperatures in the presence of yeast extract at various concentrations was investigated. As shown in Table 2, degradation occurred most rapidly at 43°C in the presence of 0.002% yeast extract, and PLA was degraded to some extent at temperatures below 37°C. A very low concentration of yeast extract (0.002%) stimulated the degradation of PLA, whereas a relatively high concentration (0.02%) suppressed it. These findings were obtained after 11 days of cultivation, and a longer cultivation period might result in further degradation, even under relatively unfitting conditions.

Time course of degradation

The time course of the degradation of PLA is shown in Fig. 1 and a photograph is shown in Fig. 2. All visible pieces of film were recovered, although some found under a microscope were not gathered. Strain No. 3118 showed lower PLA-degrading activity at the

Table 1. Identification of strain No. 3118.

Aerial mycelium	+, white	Lactose	+
Substrate mycelium	beige	D-Melibiose	+/-
Cell size	0.5 μ m (1.5–7.5 μ m long, after 2 days)	D-Melezitose	–
Spore size	0.8 $\times$ 1.2 μ m	D-Trehalose	+
Spore chains	+	Raffinose	+
Flagella of vegetative cells	–	Adonitol	–
Flagella of spores	–	Inositol	+
Growth temperature 15°C	+/-	Mannitol	+
30°C	+	meso-Erythritol	–
48°C	+	Salicin	+
53°C	–	Glycerol	+
Gram stain	+	Dextrin	+
Growth in the presence of 7% NaCl	+	Starch	+/-
Anaerobic growth	–	Inulin	+
Catalase	+	Decomposition of	
Oxidase	+	Hypoxanthine	+/-
OF Test	no change	Xanthine	–
Hydrolysis of starch	+	Benzoate	–
Hydrolysis of litmus milk	+/-	Citrate	+
Hydrolysis of gelatin	+	Whole cell sugar	
Utilization of sugars		Arabinose	+
D-Glucose	+	Galactose	+
D-Galactose	+	Diamino acid of peptidoglycan	DL-diaminopimelic acid
L-Rhamnose	+	Mycolic acid	–
D-Fructose	+/-	Menaquinone	MK-9(H ₄)
D-Ribose	–	Fatty acid pattern	branched saturated FA (16:0 iso), unsaturated FA (16:1 iso), 2-hydroxy FA (16:0 iso 2OH) and 10-methyl branched FA (17:0 10-methyl),
L-Arabinose	+/-		genus <i>Amycolatopsis</i>
D-Xylose	+		
Sucrose	+		
Maltose	+		
D-Cellobiose	+		
		16 S rDNA sequence similarity ^a	

^a The first 500 bp sequence was analyzed.

Table 2. Effects of temperature and yeast extract on the degradation of PLA film.

Yeast extract conc (%)	Loss of film weight (%)			
	30°C	37°C	43°C	48°C
0	9.2 $\pm$ 0.7	23.1 $\pm$ 3.8	58.2 $\pm$ 5.9	5.0 $\pm$ 0.1
0.002	12.7 $\pm$ 1.2	53.1 $\pm$ 4.3	85.8 $\pm$ 5.2	12.5 $\pm$ 3.4
0.02	6.7 $\pm$ 0.5	16.0 $\pm$ 2.9	49.6 $\pm$ 3.6	5.1 $\pm$ 0.2

Weight loss was measured after 11 days of cultivation. The film weight loss was 3.1 $\pm$ 0.4% after sterilization in the medium, and the film weight loss was 4.9 $\pm$ 0.5% after 11 days of incubation at 30 to 48°C in the presence of yeast extract at various concentrations without inoculation (control values). Duplicate experiments were performed.

time when the organism was initially isolated; however, its activity increased as a result of repeated selection of the strain from cultures that showed high activity. The film was originally completely transparent. It became slightly opaque on sterilization in the medium, then whitened and rough during the cultivation period. The original shape of film disappeared within 2 weeks, accompanied by some bacterial growth (OD₆₆₀=0.27

Fig. 1. Time course of degradation of PLA film at 43°C.

The medium is described in the text. Cultivation was carried out at pH 7.0 under static conditions after seeding (1.0% vol/vol) with a preculture. Symbols: ●, film wt; ■, growth. Film weight before sterilization was calculated as 100%, and the value at 0 d means weight after sterilization. The control value without inoculation was 94.9 $\pm$ 0.3% after 12 days of incubation. Error bars show the standard error of the mean of four replicates.

Fig. 2. Disintegration of PLA film during the culture. The disintegration process typically observed on cultivation at 43°C is shown.

Fig. 3. Thin-layer chromatography after incubation of the supernatant with PLA film.

Culture supernatant (at 43°C for 12 days) was concentrated and incubated with film (1%) at 43°C for 7 days. A, incubation with heat-treated supernatant (100°C, 5 min); B, incubation with supernatant; C, incubated supernatant (B) and lactic acid; D, lactic acid.

at maximum). On the contrary, maximum growth without film was 0.062 at OD₆₆₀ (after 6 days), showing that the film was utilized as a carbon source. Reeve et al. (1994) reported that degradation of a polymer consisting of 100% L-type lactic acid by proteinase K is slower than in polymers containing D-type isomer at several ratios. Furthermore, the degradation of lactic

acid-containing polymers occurs more rapidly than the degradation of homo-PLA. Since PLA, which was nearly 100% L-type, was used in our experiments, it seems likely that polylactic acids consisting of a certain percent of D-type isomers or other chemicals such as glycolic acid are presumably degraded easily and rapidly.

Products in the culture supernatant

The products generated from PLA film in the culture supernatant were analyzed. Cultivation was done with about 0.5% film. In the supernatant of 6 and 12 days cultivation, lactic acid was not detected by thin-layer chromatography or by the lactate dehydrogenase assay method. The supernatant was concentrated to about threefold, since protein in it was of low concentration (0.05 mg/ml). After incubation with film at 43°C for 7 days, lactic acid was detected (Fig. 3). Lactic acid produced was also confirmed by the lactate dehydrogenase assay method (Stolzenbach, 1966). This result indicates that the supernatant contained at least the degrading enzyme of an exotype hydrolase producing lactic acid. Finally, the strain certainly uses PLA as lactic acid.

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