Microbial Degradation of an Aliphatic Polyester with a High Melting Point, Poly(Tetramethylene Succinate)

HARDANING PRANAMUDA,¹ YUTAKA TOKIWA,^{2*} AND HIDEO TANAKA¹

Institute of Applied Biochemistry, University of Tsukuba,¹ and National Institute of Bioscience and Human-Technology,² Tsukuba, Ibaraki 305, Japan

Received 3 October 1994/Accepted 15 February 1995

The biodegradability of poly(tetramethylene succinate) (PTMS), a synthetic aliphatic polyester with a high melting point, was evaluated. The ecological study showed that the distribution of PTMS-degrading microorganisms in soil environments was quite restricted compared with the distribution of microorganisms that degrade poly(ϵ -caprolactone) (PCL), a polyester with a low melting point. However, in soil samples in which the formation of a clear zone was observed, PTMS-degrading microorganisms constituted 0.2 to 6.0% of the total number of microorganisms, which is very close to the percentage (0.8 to 8.0%) observed for PCL-degrading microorganisms. Five strains were isolated from colonies which formed distinct clear zones on agar plates with emulsified PTMS. In liquid cultures of the isolates with ground PTMS powder, strain HT-6, an actinomycete, showed the highest PTMS degrading activity. It assimilated about 60% of the ground PTMS powder after 8 days of cultivation. When a PTMS emulsion was used, a higher degradation rate was observed and more than 90% of the PTMS was assimilated in 6 days. PTMS degradation products were analyzed by gas chromatography, and it was found that 1,4-butanediol, 4-hydroxy *n*-butyrate, and succinic acid accumulated during cultivation. Degradation of PTMS film by the strain occurred in two steps: fragmentation and then the formation of hemispherical holes on the surface of the film. Strain HT-6 was also able to assimilate PCL and poly(β -hydroxybutyrate) (PHB). The crude enzyme showed a wide range of substrate specificity, being able to degrade low-molecular-weight PTMS, PCL, PHB, and even high-molecular-weight PTMS.

The disposal of nondegradable synthetic polymers has become a worldwide environmental problem, and there is a need for the development of biodegradable polymers (4, 10). Aliphatic polyesters have good biodegradability potential. Two representative polyesters, namely, poly(ϵ -caprolactone) (PCL), a synthetic polyester, and poly(β -hydroxybutyrate) (PHB), a microbial polyester, have been extensively investigated with respect to their biodegradability (1–3). These polyesters are currently produced commercially. The low melting point of PCL ($T_m = 62^\circ\text{C}$) limits the range of application of this polymer. Although the melting point of PHB is high ($T_m = 175^\circ\text{C}$), its applicability is limited because of its high crystallinity. Recently, poly(tetramethylene succinate) (PTMS), a polyester synthesized from 1,4-butanediol and succinic acid, has attracted much attention for its high melting point ($T_m = 113^\circ\text{C}$). However, it has been reported that biodegradability of synthetic polyesters by *Rhizopus arrhizus* lipase decreases with increasing melting point (10, 12). The biodegradability of polyesters with low melting points, such as poly(ethylene adipate) ($T_m = 48.5^\circ\text{C}$) and PCL, is high, while the biodegradability of polyesters with high melting points, such as PTMS, is low (10).

The evaluation of plastic polymer biodegradability has to be comprehensive in order to minimize harmful effects of biodegradation on the environment. Not only physiological studies, such as studies of enzymatic or microbial degradation and their mechanisms, but also ecological studies, such as studies of the distribution of degrading microorganisms and their ratios in the natural environment, are necessary to gain a better understanding of plastic polymer biodegradation. We previously established a method for evaluating the distribution of polymer-

degrading microorganisms in different environments and applied it to the screening of polyester-degrading microorganisms (6, 8). We found that PCL- and PHB-degrading microorganisms are distributed widely and that they represent 0.2 to 11.4% and 0.8 to 11.0% of the total number of microorganisms in the environment, respectively (6).

There are no reported studies on the biodegradability of PTMS, but there is a great need for a comprehensive study of the biodegradability of this polymer before it can be used for practical applications. In this report, we investigated the distribution of PTMS-degrading microorganisms and isolated a microbial strain with high PTMS-degrading activity. Degradation of various forms of PTMS was also investigated, and the mechanism of PTMS degradation was elucidated. Finally, we examined the abilities of the strain and its secreted enzymes to degrade other polyesters.

MATERIALS AND METHODS

Materials. Two types of PTMS with different molecular weights were used. The chemical structure of PTMS is shown in Fig. 1. Low-molecular-weight PTMS (PTMS-L) was obtained from Dainippon Ink & Chemicals, Inc., and was used directly without purification. To prepare high-molecular-weight PTMS (PTMS-H), polycondensation of PTMS-L by removing 1,4-butanediol was carried out at 200°C in a vacuum with isopropyl titanate(IV) as a catalyst. The polymer thus recovered was then purified by precipitation in methanol after being dissolved in chloroform. Poly-L-lactide (PLA) and PCL (TONE P-767) were obtained from Mitsui Toatsu Chemicals Inc. and Union Carbide Corp., respectively, and were used without purification. Technical-grade granules of PHB [BX GV9(EE)] were obtained from ICI (Tokyo, Japan) and were purified by precipitation with hexane after being dissolved in hot chloroform. The number-average molecular weight (M_n) and polydispersity of each polymer were estimated by gel permeation chromatography (HLC-8020; Tosoh Co.) with column systems of TSK gel 4000Hxl, 3000Hxl, 2000Hxl, and 1000Hxl. Polystyrene standard samples were used for calibration.

Soil samples. Forty-five soil samples (taken at about 5 cm below the soil surface) were collected from various places in Tsukuba City, such as paddy fields, weed fields, roadsides, and dumping grounds, etc., between June and October 1993.

* Corresponding author. Mailing address: National Institute of Bioscience and Human-Technology, 1-1 Higashi, Tsukuba, Ibaraki 305, Japan. Phone: 81-298(54)6089. Fax: 81-298(54)6009.

FIG. 1. Chemical structure of PTMS.

Culture media. Preparation of basal medium containing 0.1% (wt/vol) emulsified polyester was essentially the same as described in our previous report (6). Nutrient broth medium was used for purification of the isolates, and ISP 2 medium, composed of 1.0% malt extract, 0.4% yeast extract, and 0.4% glucose (pH 7.0), was used for purification of the actinomycete cultures.

PTMS-L powder was prepared by grinding the PTMS-L solid in a mortar and then sieving the powder through a screen with a pore size of 180 μm . The powder was sterilized by heating at 105°C for 24 h.

PTMS-H film was prepared by the heat-press method at 150 kgf/cm² and 150°C. The film (about 44 μm thick) was sterilized with 5% (wt/vol) sodium hypochlorite for a few seconds.

Cell cultures. Soil samples were diluted 1/10³ to 1/10⁵ with sterilized water. A 0.2-ml aliquot was spread on an agar plate containing emulsified PTMS or PCL. The cultures were incubated at 30°C. The total colonies and clear zones on the agar plates were enumerated within 30 days. The numbers represent total microorganisms and polyester-degrading microorganisms, respectively. The numbers of colonies and clear zones were expressed as CFU or clear-zone-forming units per gram of soil sample. One colony with a distinct clear zone on the PTMS plate was isolated and purified several times using the basal and nutrient broth media.

Liquid cultures of the isolates were grown in 500-ml Erlenmeyer flasks containing 100 ml of the basal medium. The flasks were incubated on a rotary shaker (180 rpm) at 30°C. Culturing was done in triplicate, and the mean value for each experiment was used.

Crude enzyme from the isolate. Culture broth obtained after 8 days of liquid culture with PTMS emulsion was centrifuged at 4,500 $\times$ g, and the supernatant was employed as crude enzyme. The clear-zone-forming ability of the crude enzyme was examined with a penicillin cup. The heat-sterilized cup was put on an agar plate containing emulsified polyester and was filled with 0.2 ml of crude enzyme filtered aseptically through a Millipore filter (0.22- μm pore size). The plates were incubated at 30°C for 2 days.

Analysis of PTMS degradation products. Before the extraction of residual PTMS, a 5-ml sample was taken from the culture broth for analyses of water-soluble total organic carbon (TOC), degradation products, and pH. TOC was measured with a TOC-5000 analyzer (Shimadzu Corp.). The degradation products were analyzed by gas chromatography (Shimadzu GC 14B) with the following equipment and operating conditions: column, Tenax (2.1 m by 3.2 mm inside diameter); carrier gas, nitrogen (1.5 atm); injector temperature, 270°C; oven temperature, 210°C; flame ionization detector temperature, 270°C; sample size, 5 μl ; and internal standard, 1,6-hexanediol. Under these conditions, peaks for 1,4-butanediol, 4-hydroxy *n*-butyrate, succinic acid, and 1,6-hexanediol were detected at retention times of 2.0, 2.4, 3.9, and 5.0 min, respectively. The concentration of each degradation product was expressed after TOC conversion.

Recovery of residual PTMS and cells from the culture broth. Residual ground PTMS powder was recovered directly from the culture broth by extraction with chloroform. Since the emulsion of polyester was so stable that it could not be extracted directly with chloroform, 100 ml of ethyl acetate was added in advance and the emulsion was heated in boiling water for 3 min to disrupt the micelle structure of the surface-active agent. After that, 100 ml of chloroform was added. The extraction was conducted three times. The recovered polymer was dried at 80°C in a vacuum overnight. Under these conditions, PCL, PHB, and PTMS emulsions were efficiently recovered, but the rate of recovery of PLA emulsion was low.

The cells were collected by centrifugation at 4,500 $\times$ g for 10 min and dried at 105°C for 16 h.

Scanning electron microscope observations. PTMS-H film samples taken after the microbial degradation were observed under a JEOL scanning electron microscope, model JSM-T220, with 15-kV acceleration.

RESULTS

Comparison of the distribution of PTMS- and PCL-degrading aerobic microorganisms in soil environments. It was confirmed that among 45 soil samples investigated, the clear zones were observed in only 19 samples on PTMS-L plates versus 33 samples on PCL plates. In the samples in which the formation of a clear zone was observed on both kinds of plates, the

TABLE 1. Isolates and their ability to assimilate ground PTMS-L powder^a

Isolate	Organism	Amt of PTMS-L assimilated (mg) ^b	Cell dry wt (mg) ^c
HT-3	Mold	14.3 $\pm$ 1.0	8.2 $\pm$ 0.5
HT-4	Bacterium	12.9 $\pm$ 0.4	6.3 $\pm$ 0.3
HT-6	Actinomycete	59.6 $\pm$ 0.9	20.2 $\pm$ 1.0
HT-8	Bacterium	9.3 $\pm$ 0.5	5.4 $\pm$ 0.1
HT-33	Mold	16.2 $\pm$ 0.8	9.3 $\pm$ 0.7

^a The isolates were cultivated in 100 ml of medium containing 100 mg of ground PTMS-L powder on rotary shakers (180 rpm) at 30°C for 8 days.

^b Values are means $\pm$ standard errors of the means.

^c Values are means $\pm$ standard errors of the means.

number of clear zones was about 10⁵ to 10⁷/g of sample, and the percentages of clear zones relative to the total number of colonies on PTMS-L and PCL plates were estimated to be in the ranges of 0.2 to 6.0% and 0.8 to 8.0%, respectively.

Isolation of PTMS-degrading microorganisms. We succeeded in isolating five PTMS-degrading strains, as shown in Table 1. It was confirmed that all the isolates formed clear zones on both PCL and PTMS-L plates. In order to assess the ability of the strains to degrade PTMS, liquid cultures were grown with ground PTMS-L powder as a substrate. The rate of cell growth and degree of PTMS-L assimilation were highest for strain HT-6. Of the 100 mg of PTMS-L initially added, 60% was assimilated, and about 20 mg of cells was formed after 8 days of cultivation. We repurified strain HT-6 with ISP 2 medium to get a single colony of the strain and used it for subsequent investigations of PTMS degradation. Microscopic observation revealed that strain HT-6 is likely to be an actinomycete. A photograph of strain HT-6 colonies with clear zones is shown in Fig. 2. Taxonomic identification of the strain is now in progress.

Degradation of PTMS-L ground powder and emulsion by strain HT-6. Time courses of PTMS-L degradation by strain HT-6 are shown in Fig. 3. When ground PTMS-L powder was used as a substrate (Fig. 3a), rapid degradation of PTMS-L and a remarkable rise in TOC were observed within 4 days of cultivation. After that time, the degradation became slower, while the TOC decreased gradually. Even at 10 days of cultivation, about 40% of the PTMS-L still remained in the culture broth, while about 20 mg of cells was formed. On the other hand, when PTMS-L was emulsified into the medium (Fig. 3b), degradation occurred more rapidly and 90% degradation was

FIG. 2. Strain HT-6 colonies on an agar plate with emulsified PTMS.

FIG. 3. Degradation of PTMS-L by strain HT-6 with ground PTMS powder (a) and PTMS emulsion (b) as substrates.

achieved in 6 days of cultivation. In both cultures, the pH dropped slightly during cultivation.

Degradation of PTMS-H emulsion. In order to investigate the effect of the molecular weight of PTMS on degradation by strain HT-6 and to elucidate the mechanism of degradation, liquid cultures were grown in a medium containing PTMS-H emulsion. Figure 4 illustrates the time course of degradation and the release of degradation products, which were determined by gas chromatography. The degradation of PTMS-H by strain HT-6 occurred as rapidly as the degradation of PTMS-L (Fig. 3b). After 4 days of cultivation, about 80% of the PTMS was consumed, and the accumulation of 4-hydroxy *n*-butyrate was observed. At 12 days of cultivation, most of the degradation products were assimilated completely by the strain, and about 30 mg of cells was recovered with a cell yield ($Y_{x/s}$) of

FIG. 4. Degradation of PTMS-H emulsion by strain HT-6. Symbols: ●, PTMS recovered; ○, cell growth; □, TOC; ▴, 1,4-butanediol; ■, succinic acid; ▴, 4-hydroxy *n*-butyrate; △, pH.

0.26 mg of cells per mg of substrate (s , amount of substrate consumed; x , amount of cell growth formed). During cultivation there was no significant drop in pH.

Degradation of PTMS-H film. To examine the degradability of PTMS-H film, liquid cultures were grown with 100 mg of film as a substrate. It was confirmed that at 4 days of cultivation, the film started to disintegrate, and at 6 days of cultivation, the fragments of the film were finely dispersed in the culture broth. The surface morphology of the film was observed with a scanning electron microscope at various stages of cultivation (Fig. 5). Small cracks on the film were formed at 2 days of cultivation, and the cracks became larger at 4 days of cultivation, thereby causing the fragmentation of the film. Hemispherical holes 16 to 20 μ m in diameter were observed on the surface of the film fragments at 6 days of cultivation. In comparison with the degradation of PTMS emulsion or ground powder, degradation of PTMS-H film occurred very slowly.

Degradation of other polyesters. The ability of strain HT-6 to degrade other polyesters was investigated. Five kinds of polyesters (PLA, PHB, PCL, PTMS-L, and PTMS-H) were used in this experiment. Gel permeation chromatography revealed that all polymers showed a single peak with no shoulders. The M_n and polydispersity (M_w/M_n) are shown in Table 2. Clear-zone formation on agar plates by colonies and crude enzyme was observed, and liquid cultures were grown on each polyester emulsion. Both the colonies and the crude enzyme secreted by strain HT-6 formed clear zones on all plates except the PLA plate. Moreover, strain HT-6 assimilated more than 90% of each polyester after 8 days of cultivation in the liquid cultures.

DISCUSSION

Two possible methods of treating biodegradable PTMS plastic wastes are (i) disposing of wastes directly in the environment and (ii) treating them in a closed system before disposing of them in the environment. Our ecological study showed that in comparison with the distribution of PCL-degrading microorganisms, the distribution of PTMS-degrading microorganisms is quite restricted. However, the ratios of the degrading microorganisms to total microorganisms were almost the same for both PTMS and PCL. This suggests that, although it may take a long time, PTMS can be degraded in the natural environment. Treatment in closed systems can be achieved by cultivating PTMS-degrading microbial strains. Strain HT-6 has good potential for treatment of PTMS, since it can degrade and assimilate various forms of PTMS, including film, which is the form that will be most commonly used. The results presented here suggest that in a liquid culture of strain HT-6, PTMS was first decomposed into water-soluble substances, such as 1,4-butanediol, 4-hydroxy *n*-butyrate, and succinic acid, and then assimilated by the cells. On the other hand, the degradation of film by the strain occurred in two steps, namely, the fragmentation and the formation of hemispherical holes on the film surface (Fig. 5). There was no significant pH drop during cultivation, since the accumulation of acid substances such as succinic acid was not significant (Fig. 4).

The cells recovered after culturing had a low cell yield ($Y_{x/s}$) of 0.25 to 0.28 mg of cells per mg of PTMS. The residual polyester recovered from the culture broth may contain other substances, such as the lipid layer of the cells. Hence, the low cell yield might be due to the removal of the lipid layer from the cells during extraction.

The rate of degradation of PTMS by strain HT-6 in liquid culture depended on the form of PTMS substrate used. The rate of degradation was highest for the emulsion, lower for the

FIG. 5. Surface morphology of PTMS films after degradation by strain HT-6 on day 0 (a), day 2 (b), day 4 (c), and day 6 (d).

ground powder, and lowest for the film. PTMS emulsion was degraded rapidly and almost completely because of its very small particle sizes and large surface area. On the other hand, the degradation of PTMS ground powder and film was comparatively low because of its larger particle sizes. The surface area of the polymer is thus an important factor in determining the rate of microbial degradation. It has been reported that crystal structure and molecular weight influence the microbial degradability of polymers (5, 7). The emulsification of PTMS might change the crystal structure so that the size of spherulite, the rounded aggregate of crystals which is the less-degraded region of the polymer, becomes smaller and consequently more susceptible to microbial attack. In contrast, the low rate of degradation of PTMS film was due to the highly developed crystal structure in the film.

No effect of molecular weight on the degradation was observed, as strain HT-6 could degrade PTMS-H as rapidly as PTMS-L (Fig. 3b and 4).

Lipase is known to be able to hydrolyze aliphatic polyester (11, 12). PHB depolymerase, the PHB-hydrolyzing enzyme, is not able to hydrolyze PCL, and conversely, lipase cannot hydrolyze PHB (9). As shown in Table 2, both the colony and the crude enzyme were able to form clear zones on PCL, PTMS (either low or high molecular weight), and PHB plates, suggesting that the enzymes produced by strain HT-6 have a wide

range of substrate specificity. Further study is needed to determine whether the enzymes produced are composed of two or more types of enzyme, thus enabling degradation of PCL, PTMS, and PHB.

TABLE 2. Degradation of various polyesters by strain HT-6^a

Polyester	M_n	M_w/M_n^b	Clear zone formed by:		Liquid culture ^c	
			Colony	Enzyme	Amt of polyester assimilated (mg)	Cell dry wt (mg)
PLA	3.43×10^3	1.12	—	—	ND ^d	14.0 ± 1.6
PHB	1.45×10^5	1.73	+	+	94.3 ± 0.7	21.8 ± 1.9
PCL	6.73×10^4	2.01	+	+	90.7 ± 0.9	14.1 ± 1.1
PTMS-L	6.14×10^3	1.32	+	+	93.6 ± 0.9	22.0 ± 1.6
PTMS-H	5.74×10^4	1.47	+	+	94.6 ± 0.9	25.2 ± 0.9

^a Strain HT-6 was cultivated in 100 ml medium containing 100 mg of emulsified polyester on a rotary shaker (180 rpm) at 30°C for 8 days.

^b Polydispersity.

^c Values are means $\pm$ standard errors of the means.

^d ND, not detected.

REFERENCES

1. **Benedict, C. V., W. J. Cook, P. Jarrett, J. A. Cameron, S. J. Huang, and J. P. Bell.** 1983. Fungal degradation of polycaprolactones. *J. Appl. Polym. Sci.* **28**:327–334.
2. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1990. Plastic from bacteria and for bacteria: poly(β -hydroxyalkanoates) as natural, biocompatible, and biodegradable polyesters. *Adv. Biochem. Eng. Biotechnol.* **41**:77–93.
3. **Cameron, J. A., C. L. Bunch, and S. J. Huang.** 1988. Microbial degradation of synthetic polymers, p. 553–561. *In* D. R. Houghton, R. N. Smith, and H. O. W. Egging (ed.), *Biodeterioration*, 7th ed. Elsevier Applied Science, London.
4. **Diamond, M. J., B. Freedman, and J. Garibaldi.** 1975. Biodegradable polyester films. *Int. Biodeterior. Bull.* **11**:127–132.
5. **Jarrett, P., C. V. Benedict, J. P. Bell, J. A. Cameron, and S. J. Huang.** 1984. Mechanism of the biodegradation of polycaprolactone, p. 181–192. *In* S. W. Shalaby, A. S. Hoffman, B. D. Ratner, and T. A. Hirbert (ed.), *Polymers as biomaterials*. Plenum Press, New York.
6. **Nishida, H., and Y. Tokiwa.** 1993. Distribution of poly (β -hydroxybutyrate) and poly (ϵ -caprolactone) aerobic degrading microorganisms in different environments. *J. Environ. Polym. Degrad.* **1**:227–233.
7. **Nishida, H., and Y. Tokiwa.** 1993. Effects of higher-order-structure of poly(3-hydroxybutyrate) on its biodegradation. II. Effects of crystal structure on microbial degradation. *J. Environ. Polym. Degrad.* **1**:65–80.
8. **Nishida, H., and Y. Tokiwa.** 1994. Confirmation of anaerobic poly (2-oxepanone) degrading microorganisms in environments. *Chem. Lett.* **1994**:1293–1296.
9. **Tanio, T., T. Fukui, Y. Shirakura, T. Saito, K. Tomita, T. Kaiho, and S. Masamune.** 1982. An extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. *Eur. J. Biochem.* **124**:71–77.
10. **Tokiwa, Y., T. Ando, T. Suzuki, and K. Takeda.** 1990. Biodegradation of synthetic polymers containing ester bonds, p. 136–148. *In* J. E. Glass and G. Swift (ed.), *Agricultural and synthetic polymers*. American Chemical Society, Washington, D.C.
11. **Tokiwa, Y., and T. Suzuki.** 1978. Hydrolysis of polyesters by *Rhizopus delemar* lipase. *Agric. Biol. Chem.* **42**:1071–1072.
12. **Tokiwa, Y., T. Suzuki, and K. Takeda.** 1986. Hydrolysis of polyesters by *Rhizopus arrhizus* lipase. *Agric. Biol. Chem.* **50**:1323–1325.