Poly(L-lactide)-Degrading Activity of a Newly Isolated Bacterium

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ABSTRACT: A new strain exhibiting poly(L-lactic acid) (PLLA)-degrading activity was isolated from the compost loaded with low-molecular weight PLLA. The strain was rod-shaped gram positive bacterium and was identified to be *Bacillus licheniformis* by the 16S rDNA sequence analysis. It degraded not only low-molecular weight PLLA but also other PLLAs having higher molecular weight. The lower the molecular weight of PLLA, the faster the biodegradation rate. And thereby 45% and 81% of PLLAs with weight average molecular weight of 256,000 and 5,000, respectively, were metabolized into CO_2 after 40 days of biodegradation at 58°C in the sterilized compost inoculated with the bacterium. According to the 16S rDNA sequence analysis, *B. licheniformis* PLLA-2 belonged to *Bacillus* spp. phylogenetically. However, there existed almost no phylogenetic relationship between *B. licheniformis* PLLA-2 and the previously reported PLLA-degrading strains. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 234–239, 2008

Key words: biodegradation; *Bacillus licheniformis* PLLA-2; poly(L-lactic acid); molecular weight; polyesters

INTRODUCTION

Poly(L-lactic acid) (PLLA), an aliphatic polyester, has recently drawn a lot of attention because of the fact that it is biodegradable and can be produced from renewable resources. Owing to the recent formidable efforts to reduce the production cost[1,2] and to improve the physical properties, PLLA is finding applications to substitute many commodity plastics, especially single-use packaging materials aside from the niche applications such as biomedical materials.[3] However, it has been known that PLLA can be degraded only by a limited number of microorganisms as listed in Table I and sometimes PLLA buried in soil remains intact for many years. Therefore, it is important to explore new PLLA-degrading microorganisms and to investigate biodegradation of PLLA to minimize the environmental impact coming from mass consumption of these plastics in a near future.

Fungi such as *Fusarium moniliforme* and *Penicillium roqueforti* can utilize PLLA oligomers,[4] while another fungus *Tritirachium album* obtained from American Type Culture Collection (ATCC 22563) can degrade high-molecular-weight PLLA.[5] *Amycolatopsis* spp.[6] and *Saccharothrix* spp.[7] belonging to Actinomycetes have also been reported to be PLLA degraders. The thermophilic bacteria such as *Bacillus smithii*,[8] *Bacillus brevis*,[9] *Geobacillus thermocatenulatus*,[10] and *Paenibacillus amylolyticus*,[11] were also found to be active for the PLLA degradation.

In this study, isolation of microorganisms was attempted from the compost made from animal fodder to explore a new strain capable of degradation of PLLA. The isolated strain was identified by using the 16S rDNA sequence analysis and its phylogenetic relationship with other PLLA-degrading strains was examined.

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MATERIALS AND METHODS

Enrichment medium

The composition of the enrichment medium for cultivation was as follows (g/L): $(NH_4)_2SO_4$, 4; K_2HPO_4, 2; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; and yeast extract, 0.06. The final pH was adjusted to be 7.0.[9,10]

Preparation of emulsified PLLA

Emulsions were prepared by using a detergent (Plysurf A210G). PLLA (1 g) was dissolved in 40 mL of dichloromethane. The PLLA solution was poured into 1,000 mL of basal medium containing 0.1g of the detergent and blended by using an ultrasonicator (280 W, 90 min). After emulsification, dichloromethane was removed by devolatilization at 80°C for 3 h.[12]

TABLE I
Microorganisms Capable of PLLA Degradation Reported in the Literatures

Microorganisms	References
Fusarium moniliforme (fungi)	[4]
Penicillium roqueforti (fungi)	[4]
Tritirachium album (fungi)	[5]
Amycolatopsis sp. (actinomycetes)	[6]
Saccharothrix spp. (actinomycetes)	[7]
Bacillus smithii (bacteria)	[8]
Bacillus brevis (bacteria)	[9]
Geobacillus thermocatenulatus (bacteria)	[10]
Paenibacillus amylolyticus (bacteria)	[11]

The composition of the basal medium was as follows (g/L): PLLA powder 1; K_2HPO_4, 2.34; KH_2PO_4, 1.33; $MgSO_4.7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0; NaCl, 0.5; yeast extract, 0.06 and 1.0 mL of trace element solution per liter of distilled deionized water (pH 7.0). The trace element solution contained 11.9 mg of $CoCl_2$, 11.8 mg of $NiCl_2$, 6.3 mg of $CrCl_2$, 15.7 mg of $CuSO_4$, 0.97 g of $FeCl_3$, 0.78 g of $CaCl_2$, and 10.0 mg of $MnCl_2$ per liter of distilled deionized water. For a solid medium with the emulsified polymer, agar (15 g/L) was added to the basal medium.[11–13]

Isolation of PLLA-degrading microorganisms

Compost, made from animal fodder, was a source to screen PLLA-degrading bacteria.[11,12] The compost sample (10 g) and distilled water (100 mL) were mixed for 10 min, then the suspension solution was allowed to settle down for 30 min. The supernatant was pipetted (0.5 mL) and inoculated to enrichment medium (10 mL).[13] About 0.05 g of PLLA was added to the enrichment medium and incubated at 58°C in a reciprocal shaker (120 oscillations per minute). Subcultures were made 4 times by taking 0.5 mL of the original culture and inoculating it into a fresh medium. The enriched culture broth was spread on emulsified PLLA (1 g/L) agar plates. PLLA-degrading microorganisms were isolated from the colonies forming clear zone on the agar plate with emulsified PLLA. The isolates were stored at −80°C.[9,12]

TABLE II
Characteristics of PLLA

Sample code	M_n[a]	M_w[b]	Remarks
P5,000	2,400	5,000	Polycondensation time: 8 h
P11,000	4,000	11,000	Polycondensation time: 22 h
P34,000	6,100	34,000	Polycondensation time: 40 h
P256,000	142,000	256,000	Commercial product

[a] Number average molecular weight.
[b] Weight average molecular weight.

Synthesis of poly(L-lactic acid)

L-lactic acid (200 mL) was added to a 500 mL three neck reactor. To remove water, the reactor was immersed in an oil bath at 100°C with nitrogen flow for 1 h. The reactor was heated to 180°C under mechanical stirring at 300 rpm. Esterification was proceeded by adding titanium (IV) butoxide (TNBT, 0.08 mL) as a catalyst for 3 h, and then the reactor was evacuated by reducing the pressure to 1 torr step by step. The polycondensation reaction was continued at 1 torr and 180°C for different reaction time. The polycondensation reaction for 8, 22, and 40 h yielded PLLA having weight average molecular weight (M_w) of 5,000 (P5,000); 11,000 (P11,000); and 34,000 (P34,000), respectively (Table II).

Molecular weights were measured by gel permeation chromatography (GPC, Waters 410 detector) using polystyrene as the standard.

Preparation of compost

Animal fodder and saw dust mixture were composted in a laboratory-scale composting reactor, 50 cm in diameter and 120 cm in depth. Moisture content was controlled to be around 65%. Air was supplied to the composting mass at 1–15 L/min. The temperature during the composting was controlled not to exceed 58°C.

Biodegradation test

Biodegradation test in the compost using the laboratory-scale reactor was conducted according to KS M3100-1:2002; MOD ISO 14855:1999.[14] The air flow rate was controlled at 40 mL/min. The mature compost was sterilized at 121°C for 30 min and dried at 105°C for 24 h. After the sterilization, the compost

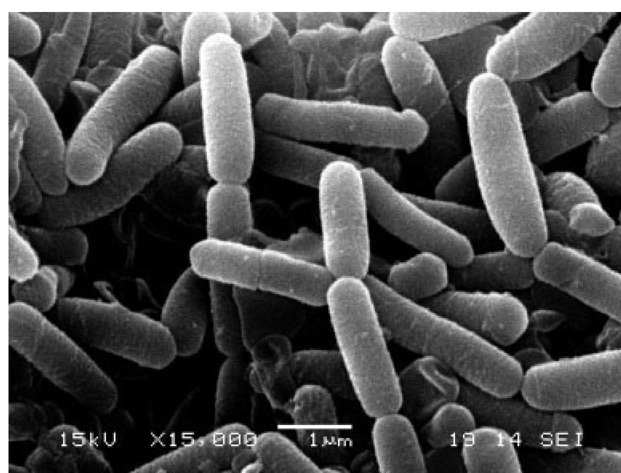

Figure 1 Scanning electron micrograph of the bacterium active for degradation of PLLA (×15,000).

(10 g) was added to sterilized distilled water (100 mL), and mixed vigorously. The supernatant was spread on plate count agar, nutrient agar, actinomycete isolation agar, and Sabouraud dextrose agar and incubated at 37°C for 3 days. Neither bacteria nor actinomycetes nor fungi grew on the agar plates indicating that the compost was completely sterilized.

A mixture of the mature compost (200 g, wet weight) inoculated with 10^{10} cfu/g of the isolated strain and test materials (5%, on dry basis) was introduced and incubated at 58°C. Moisture content was controlled to be 65%. CO_2 produced from the reactor was absorbed by 0.4*N* potassium hydroxide and 2*N* barium chloride solution, and the amount was determined by titrating the solution with 0.2*N* HCl.

Scanning electron microscopy

The morphology of PLLA film biodegraded by *B. licheniformis* PLLA-2 was examined using a SEM microscope (JEOL, model JSM-5600LV). Prior to the analysis, the sample was coated with gold to protect the sample morphology against the electron beam.

Phylogenetic analysis of 16S rDNA from *Bacillus licheniformis* PLLA-2

16S rDNAs were amplified by using universal primers fD1and rP2.[15] Their nucleotide sequences were determined with Applied Biosystems 3100 sequencer.

The sequences were aligned together with those of representative members of selected genera by using the CLUSTAL W program.[16] The evolutionary tree for the data sets was inferred from the neighbor-joining method of Saitou and Nei[17] by using the MEGA version 3.1.[18] The stability of the relationships was assessed by performing the bootstrap analyses of the neighbor-joining data based on 1000 trials.

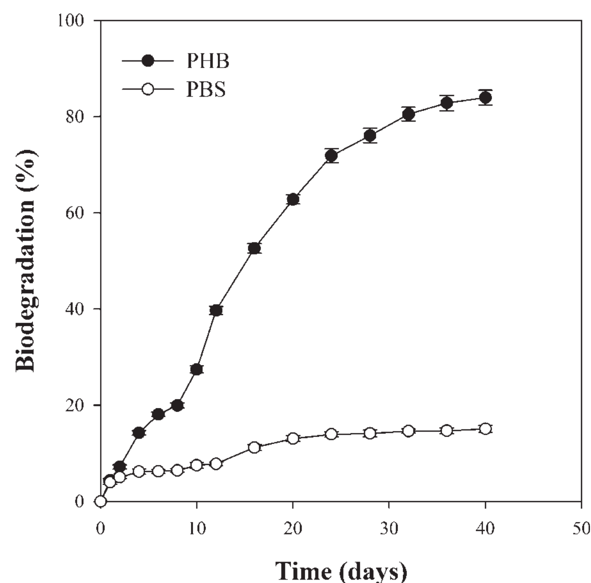

Figure 3 Degradation of poly(3-hydroxy butyrate) and poly(butylene succinate) at 58°C in the compost inoculated with the new PLLA-degrading strain.

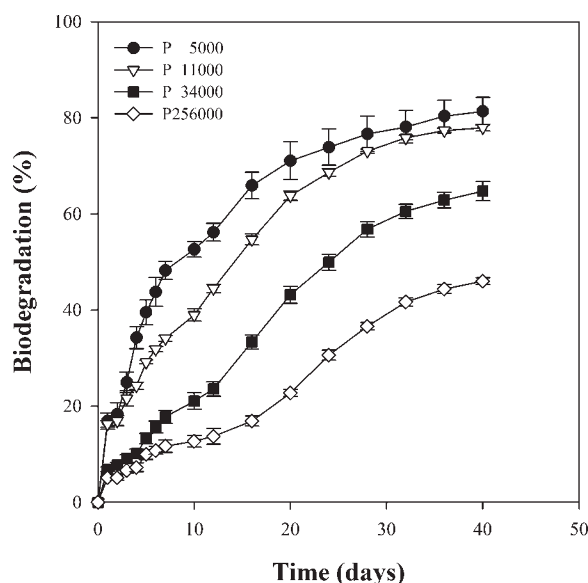

Figure 2 Biodegradation of PLLA in the compost inoculated with the new bacterium showing PLLA-degrading activity. ● P5,000; ▽ P11,000; ■ P34,000; ◇ P256,000.

Nucleotide sequence accession number

Nucleotide sequence of *B. licheniformis* PLLA-2 16S rDNA gene was deposited in the GenBank nucleotide sequence database under accession number of DQ480087.

RESULTS AND DISCUSSION

Isolation of PLLA-degrading microorganisms

PLLAs having weight average molecular weights (M_w) in the range of 5,000–34,000 were synthesized by controlling the reaction time during the polycondensation step. A stain having PLLA-degrading activity was isolated at 58°C from the compost made from animal fodder. A rod-shaped bacterium, as exhibited in Figure 1, formed clear zone on an agar plate emulsified with P5,000. The PLLA-degrading activity of the bacterium was raised through repeated enrichment cultures in the medium containing P5,000. The clear zone diameter increased from 8 to 10 mm as a consequence of 5 times of the acclimation.

Biodegradation test

The bacterium was inoculated to the compost after sterilization at 121°C for 30 min and drying at 105°C

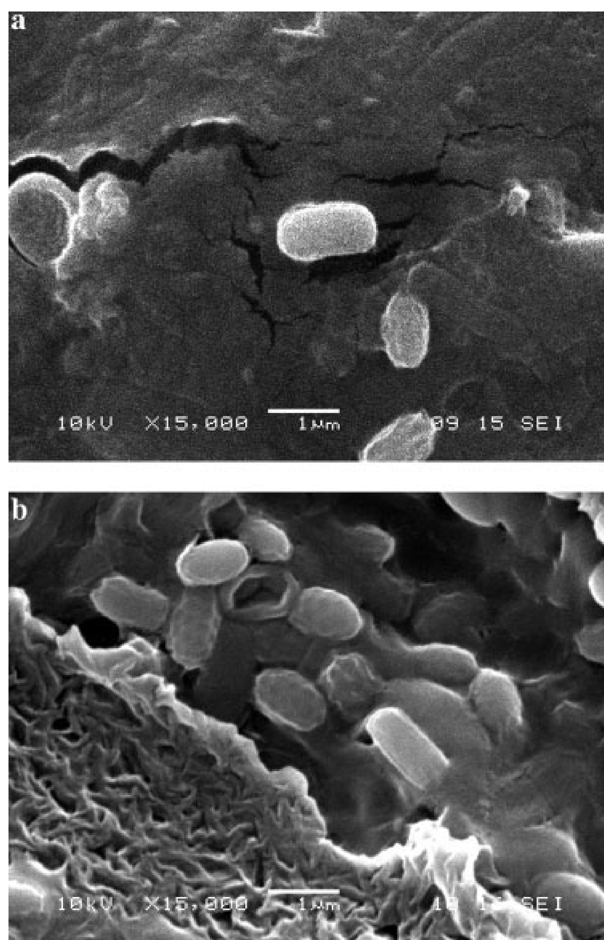

Figure 4 Scanning electron micrographs of P256,000 after degradation in the sterilized compost inoculated with the new PLLA-degrading strain (×15,000). (a) After 10 days of degradation, (b) After 20 days of degradation.

for 24 h, and then its PLLA degradation activity at 58°C was assessed by measuring the amount of CO_2 evolved from the vessel containing 3.5 g of PLLA and 200 g of the compost. The moisture content of the compost was controlled to be 65% and air was blown into the vessel at 40 mL/min during the biodegradation test.

Figure 2 demonstrates the biodegradation (%) of PLLA as a function of time. The biodegradation (%) means the cumulative amount of CO_2 divided by the theoretical amount of CO_2 assuming that all the carbon in the polymer sample was metabolized into CO_2.

It can be seen that the bacterium degraded not only P5,000 but also other PLLAs having higher M_w. The biodegradation rate of PLLA decreased monotonously as the M_w of PLLA rose. After 40 days of the biodegradation test, 81%, 78%, and 65% of the polymeric carbons were metabolized into CO_2 when the M_w of PLLAs were 5,000; 11,000; and 34,000, respectively. In case of the commercially produced PLLA, whose M_w was 256,000, the biodegradation reached 45% after the same period of the biodegradation test.

The enzymatic degradation of poly(3-hydroxyalkanoate) (PHA) took place randomly along the main chain because the plot of the ratio of the initial number average molecular weight (M_n) of PHA to the actual M_n was linear with respect to the degradation time. In contrast, the same plot for the enzymatic degradation of PLLA was not linear, indicating that the sequential chain cleavage, i.e. unzipping, as well as the random degradation occurs during the enzymatic degradation of PLLA.

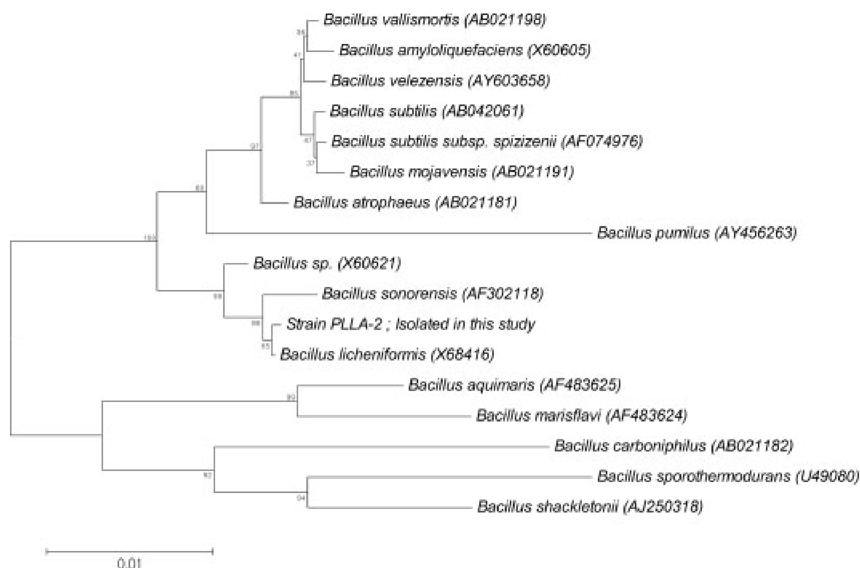

Figure 5 Phylogenetic position of the newly isolated PLLA-degrading bacterium. DNA distances were established by using the neighbor-joining method. The scale bar indicates 0.01 substitution per nucleotide position. The numbers at the branch nodes are bootstrap values from 1,000 bootstrap trials.

TABLE III
Biochemical Characteristics of *Bacillus licheniformis* PLLA-2

Characteristics	*Bacillus licheniformis* PLLA-2
Colony morphology	Irregular
Cells	
Shape	Rod
Size (μm)	(0.5–1.0) × (1.0–2.0)
Gram stain	+
Growth in enrichment media	
Nutrient agar	+
Tryptic soy agar	+
NO_3 reduced to NO_2	+
Acid from glucose	−
Hydrolysis of	
Starch	+
Casein	+
Catalase test	+
Voges-Proskauer test	−
Utilization of	
Arabinose	+
Dextrin	+
Esculin	+
Inulin	−
Mannose	+
Melibiose	+
Raffinose	−
Sorbitol	−
Xylose	−
Cellulose	+
Sucrose	+

If the degradation of polymer molecules takes place solely in a random manner, the evolution of CO_2 should wait until the polymer molecules become small enough to be absorbed into the microbial cell so that the polymeric carbons are to be metabolized into CO_2.

As disclosed in Figure 2, CO_2 evolved immediately after the onset of the biodegradation test. This can be attributed to the small molecules produced by the sequential chain cleavage from the chain end of PLLA. The lower the M_w of PLLA, the larger the number of chain ends, hence the more CO_2 evolved at the initial stage of degradation.

Figure 3 reveals that *B. licheniformis* PLLA-2 also degraded poly(3-hydroxy butyrate) (PHB) and poly (butylene succinate) (PBS). However, biodegradation rate of PBS was much slower than that of PHB as was the case by many other microorganisms.

Figure 4 demonstrates that the morphology of P256,000 degraded in the sterilized compost inoculated with *B. licheniformis* PLLA-2. P256,000 were buried in the compost, and incubated at 58°C for 20 days. Figure 4(A) exhibits the morphology of P256,000 after 10 days of biodegradation. Cracks began to form on the P256,000 surface. As the biodegradation period was extended to 20 days, the P256,000 surface was seriously eroded as shown in Figure 4(B). A lot of the rod-shaped bacteria were observed adhering firmly in the crack hollow.

Identification of PLLA-degrading microorganism

The isolated bacterium was identified to be *B. licheniformis* PLLA-2 by the 16S rDNA sequence analysis with 99% similarity. To our best knowledge, the present study reports for the first time that *B. licheniformis* PLLA-2 is one of the rare PLLA-degrading microorganisms.

The sequence analysis results of 16S rDNA are shown in Figure 5. Its biochemical characteristics are summarized in Table III.

Phylogenetic position of the newly isolated *B. licheniformis* PLLA-2 was compared with other PLLA-degrading bacteria in Figure 6 and Table IV. The 16S rDNA sequences of the previously reported PLLA-degrading bacteria were used from the type strains that were registered at Ribosomal database project (http://rdp.cme.msu.edu) and GenBank (http://www.ncbi.nlm.nih.gov). *B. licheniformis* PLLA-2 possessed 91.8%, 89.6% 84.6% and 83.1% of sequence similarities to *B. smithii*, *Geobacillus thermocatenulatus*, *Brevibacillus brevis*, and

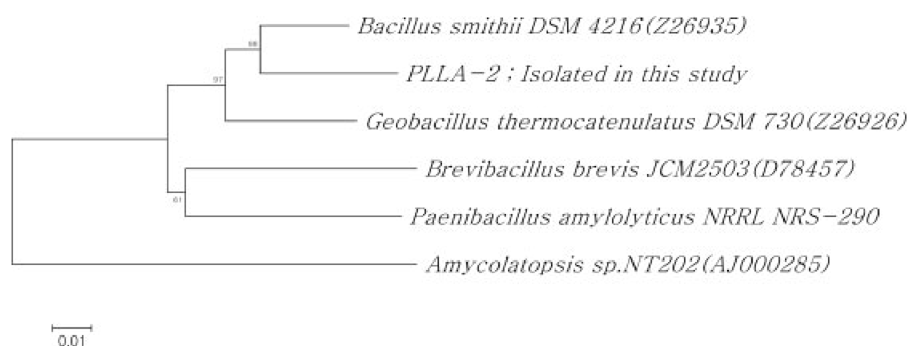

Figure 6 Phylogenetic tree based on 16S rDNA sequences comparing the isolated strain with other previously reported PLLA-degrading bacteria. DNA distances were established by using the neighbor-joining method. The scale bar indicates 0.01 substitution per nucleotide position. The numbers at the branch nodes are bootstrap values from 1,000 bootstrap trials.

TABLE IV
Homology of Sequence of 16S rDNA to Previously Reported PLLA-Degrading Str

Closet relative based on complete 16S rDNA sequence homology	Sequence similarity (%)	GenBank accession No.	References
Bacillus smittii DSM4216	91.8	Z26935	[8]
Geobacillus thermocatenulatus DSM730	89.6	Z26926	[10]
Brevibacillus brevis JCM2503	84.6	D78457	[9]
Paenibacillus amylolyticus NRRL NRS-290	83.1	D85396	[11]

Paenibacillus amylolyticus, respectively. The sequence distance between the strains reveals that there exists almost no phylogenetic relationship between *B. licheniformis* PLLA-2 and the previously reported PLLA-degrading strains.

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