Accelerated Microbial Degradation of Poly(L-lactide)

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Summary: Acceleration of the biodegradation of poly(L-lactide) (PLA) was studied. We found that the degradation rate of high molecular weight (1.3×10^5) PLA film was greatly increased by the addition of gelatin into the culture medium of the microorganisms. 100 mg of PLA film was almost completely degraded by the fungus, *Tritirachium album* (eukaryotic microorganisms), and by an actinomycete, *Saccharothrix waywayandensis* (prokaryotic microorganisms). In addition to gelatin, various insoluble proteins, peptides and amino acids also accelerate the biodegradation of PLA. Silk fibroin was the best inducer for the production of PLA-degrading enzymes of an actinomycete, *Amycolatopsis orientalis*.

Keywords: accelerated microbial degradation; biodegradable plastic; polyesters; poly(L-lactide)

Introduction

Owing to the global utilization of plastics in large quantities, their disposal as solid waste causes deleterious effects on the environment. The development of biodegradable plastics is considered to be a product innovation that can help to resolve the problems of plastic waste. Various kinds of biodegradable polyesters are presently manufactured, such as poly(L-lactide) (PLA), poly(β-hydroxybutyrate) (PHB), poly(ε-caprolactone) (PCL) and poly(butylene succinate) (PBS). PHB is a natural aliphatic polyester produced by a wide variety of bacteria as an intracellular reserve of carbon and energy. On the other hand, PCL and PBS are petroleum-based aliphatic polyesters. Although PLA is a synthetic polyester, it is regarded as a renewable bio-based plastic since its raw material, lactic acid, can be produced by fermentation of biomass, including sucrose and starch (Figure 1). Recent developments in the fermentation process of lactic acid have led to the consideration of possible mass production of PLA. It is expected that PLA produced by fermentative processes will replace many conventional plastics produced from petrochemicals.

Polymerization of PLA was described more than 70 years ago [1] but due to its relatively low molecular weight and low melting point (Tm), PLA did not attract much interest for

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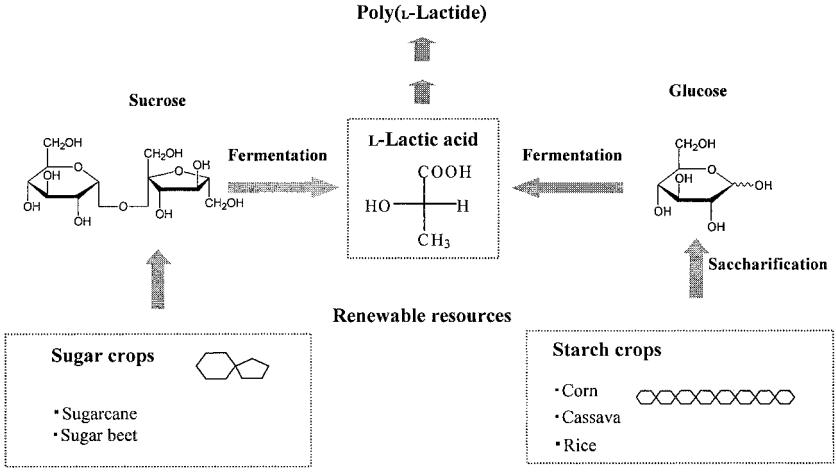

Figure 1. Production of poly(L-lactide) (PLA) from various renewable resources such as sugar and starch.

conventional applications. Several polymerization processes were then improved to obtain high-molecular weight PLA. [2-3] The mechanical properties of PLA are now sufficient to enable its use in not only medical applications but also general applications.

Hydrolytic degradability of PLA in a buffer solution has been extensively studied, especially for biomedical applications.[4-5] Implants, surgical sutures and drug delivery materials made of PLA are hydrolysable in the human body.[6] A hydrolysis study comparison of PLA and other aliphatic polyesters, e.g., PHB, PCL and PBS, indicated that at a high temperature (ca 60 °C) in a phosphate buffer (pH 7), the decrease in molecular weight of PLA was higher than those of polyesters. The initial molecular weight of PLA dropped to 50% within 14 days. [7]

The first reported enzymatic degradation of PLA used proteinase K, produced by the fungus strain *Tritirachium album.* [8] Since then this serine protease has been used for evaluating biodegradabilty and studying the degradation characteristics of PLA [9], copolymers of PLA [10] and PLA blends. [11] Not all proteases are able to degrade PLA.

The ester bond of aliphatic polyesters is susceptable to the enzymes. In general enzymatic degradability of synthetic polyesters decreases with increasing Tm. Although PHB has a high Tm of about 180 °C, it is a naturally occuring substrate and is readily degraded by various PHB depolymerase-producing microorganisms. Lipase, PHB depolymerase and

protease (proteinase K and PLA-degrading enzymes) are involved in the enzymatic degradation of polyesters. Two optically active polyesters having high Tm (ca 180 °C), PHB and PLA, are cleaved by their stereoselective enzymes, that is, PHB depolymerase and PLA-degrading enzyme, respectively. Interestingly, PLA-degrading enzymes recognize the α-bond of the stereochemical substrates, the α-amide bond of protein and the α-ester bond of PLA. Both the protein and PLA are selectively cleaved, indicating the uniqueness of the PLA-degrading enzyme. [12-14] On the other hand, lipase cleaves the ester bond randomly along the main chain of various aliphatic polyesters, which have a low Tm, for example, PCL and other polyesters having a relatively large number of methylene groups in their molecules, for example, PBS. [15-17] Aliphatic polyesters having a DL-isomer, that is, poly(DL-lactide) and poly(DL-hydroxybutyrate) are amorphous polymers. We have reported that lipase preparations from *Rhizopus arrhizus* hydrolyse both polymers. [15, 18] Among the currently known enzymes capable of degrading aliphatic polyesters, however, there are only a few reported PLA-degrading enzymes.

In 1997, the first report on microbial degradation of PLA by an actinomycete, *Amycolatopsis* strain HT-32, was published. About 60 mg of PLA film of an initial weight of 100 mg was degraded after 14 days. [19] This finding stimulated further studies on microbial degradation of PLA. Recently a PLA-degrading enzyme from the *Amycolatopsis* strain 41 was purified. [12] Interestingly, the enzyme has higher substrate specificity on PLA than proteinase K.

Microbial Degradation of PLA

We have evaluated the distribution of microorganisms capable of degrading polyesters in natural environments by the colony and clear zone counting methods using emulsified-polyester agar plates incubated at 30 °C. [20-21] The results showed that PLA-degraders have a limited distribution and scarce presence in the environment compared with those that degrade PHB, PCL and PBS. The percentages of clear-zone to total culturable colonies decreased in the order of PHB=PCL>PBS>PLA. The distribution of microorganisms capable of degrading PLA at a high temperature (50 °C) was reported to be as low as those that degrade PLA at an ambient temperature. [22] A burial test comparison of PLA and other polyesters, for example, PHB, PCL and PBS, indicated that PLA was not readily degraded when the PLA samples were buried under soil for 20 months. [23] Degradation of these polyesters in soil burial tests also decreased in the same order as found in our

investigations on the distribution of polyester-degrading microorganisms.

Thirty-nine bacterial strains of the classes *Firmicutes* and *Proteobacteria* isolated from soil samples showed the characteristic patterns of their degrading ability specific to PCL, PHB and PBS, but no PLA-degrading bacteria isolates were found. [24] Nishida et al., studied the phylogenetic relationships among the isolated strains of bacteria in the class *Proteobacteria* and *Actinobacteria*. They reported that the isolated strains were able to form clear zones on other emulsified-polyester plates but not PLA plates. [25]

We recently investigated the distribution of PLA-degraders among 41 genera (105 strains) of actinomycetes obtained from the culture collections. [26] Based on 16S rRNA gene sequence data of the investigated actinomycetes and their ability to form clear zones on PLA-emulsified agar plates, we found that the strains capable of degrading PLA phylogenetically belong to the *Pseudonocardiaceae* family and related genera, including *Amycolatopsis, Lentzea, Kibdelosporangium, Streptoalloteichus* and *Saccharothrix*. On the other hand, PCL-, PHB- and PBS-degraders are widely distributed in many families (Tokiwa & Jarerat 2003). [27] Thus, we confirmed that the *Pseudonocardiaceae* family and related genera play an important role in microbial degradation of PLA. However, it is known that these rare actinomycetes are not distributed widely in nature. This is why PLA is not readily degraded by microorganisms in the natural environment.

Accelerated Microbial Degradation of PLA

Various PLA-copolymers have been synthesized to improve the biodegradation rate of PLA by the polymerization technique. [16-17] However, the obtained copolymers generally have a Tm and molecular weight lower than PLA, and therefore some physical properties are lost.

Microbial degradation of high molecular weight (1.3×10^5) PLA film by various *Amycolatopsis* and *Saccharothrix* strains was rather limited: ca. 10 to 50 mg cast-film was degraded from the initial 100 mg film after 14 days. [26] As mentioned previously, microorganisms capable of degrading PLA have a limited distribution and scarce presence in the environment. Degradation of PLA by the selected strains was also rather limited. For the above reasons, it is very important to accelerate the biodegradation of PLA.

Designing the composition of the medium used for culturing microorganisms is one of our research strategies for accelerating the biodegradation of PLA. Gelatin was confirmed to be effective for the microbial degradation of PLA (Table 1). An addition of 0.1% (w/v)

gelatin into the culture medium resulted in the rapid degradation of high molecular weight PLA-casting films.

The time course of PLA film degradation was further investigated by culturing *S. waywayandensis* in a liquid basal medium containing 100 mg PLA film and 0.1% (w/v) gelatin. After 2 days of cultivation at 30 °C, a marked rise in cell growth was observed and more than 95 mg PLA film was degraded after 4 days of cultivation (Figure 2A). The rapid film weight loss indicates the accerelated microbial degradation of PLA. Furthermore, during the degradation, the strain also had a high ability to assimilate a monomeric degradation product of PLA, L-lactic acid. The time course of this strain in the liquid culture containing 0.1% (w/v) gelatin without film addition was simultaneously examined for comparison with the time course of film degradation (Figure 2B). The main water-soluble organic carbon (TOC) of the culture medium, that is, gelatin, dropped in parallel with cell growth, implying that the strain was able to assimilate gelatin. After

Table 1. Effect of gelatin on the accelerated degradation of PLA by *T. album* and *S. waywayandensis*.[a)]

Strains and culture media	pH	L-lactic acid	Dry cell weight (mg)	Film weight loss
T. album				
Basal medium	6 (5.8)	0 (0)	11(9)	2
Basal medium with 0.1% (w/v) gelatin	5.7(6)	14 (0)	40 (18)	76
Film control [b)]	5	0		1
S. waywayandensis				
Basal medium	7(7.2)	0 (0)	8 (4)	15
Basal medium with 0.1% (w/v) gelatin	7.5(8)	2 (0)	37(20)	95
Film control [b)]	7.2	0		2

[a)] The strains were cultured with shaking at 180 rpm and 30 °C. Culture time for *S. waywayandensis* and *T. album* were 7 and 14 days, respectively. Values in parentheses are the culture control (without film addition).
[b)] Film control was a film without cell inoculation.

cultivation for 2 days, there was no decrease in TOC and no increase in growth. Cell weight was 20 mg when cultivation was with gelatin alone. On the other hand, in cultivation with gelatin and PLA film, the dry cell weight was 40 mg. These results confirm that both the gelatin and the degradation products were assimilated by the strain.

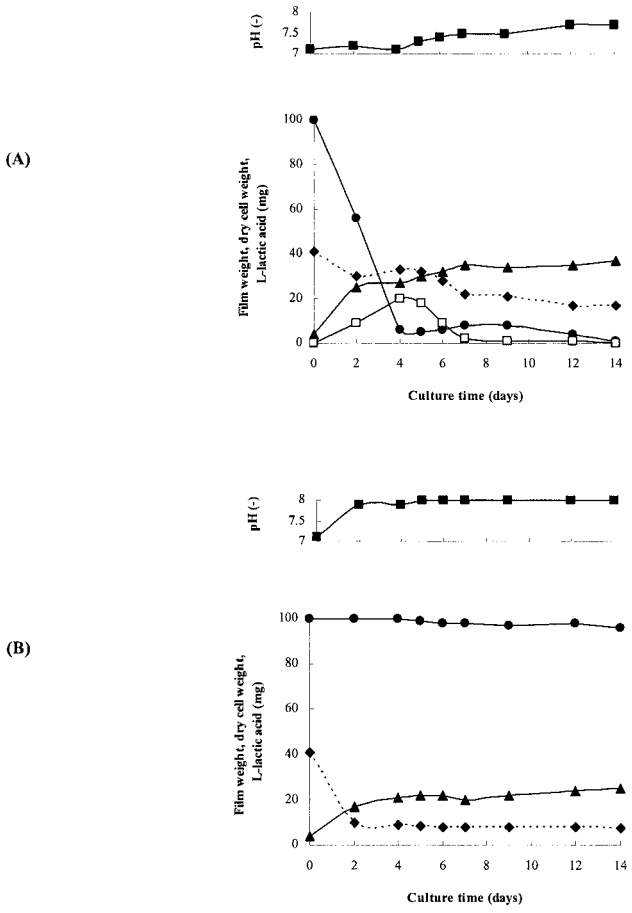

Figure 2. (A) Time course of PLA film degradation by *S. waywayandensis* in the liquid culture containing 0.1% (w/v) gelatin at 30 °C; Dry cell weight (▲), film weight (●), TOC (♦), pH (■) and L-lactic acid (□). (B) Time course of *S. waywayandensis* in the liquid culture containing 0.1% (w/v) gelatin without film addition at 30 °C; Dry cell weight (▲), TOC (♦) and pH (■). Film weight without cell inoculation (●).

No significant weight loss of PLA was observed in the case of film control without cell inoculation. Based on 16S rDNA gene sequence, *Saccharothrix waywayandensis* is now proposed as *Lentzea waywayandensis* with only 8 nucleotides (16S rDNA constituent of ca. 1400 nucleotides) different from the genus *Saccharothrix.* [28] Among the different kinds of protein, silk fibroin and elastin contain high molar ratios of glycine and alanine as found in gelatin (Figure 3).

To further examine the effect of these proteins on the accelerated biodegradation of PLA, 0.1% (w/v) protein was added into the culture medium of a fungus, *T. album,* and 2 strains of actinomycetes, *S. waywayandensis* and *Amycolatopsis orientalis.* Enzymatic degradation of PLA powder was then carried out using the culture broth as a crude enzyme. Various peptides of glycine and alanine were also investigated since they are the main amino acid components of silk fibroin, gelatin and elastin.

Areas enclosed by dark and light shades indicate insoluble proteins and peptides, respectively (see Figure 4). Gelatin is a soluble protein.

In addition to gelatin, we found that other proteins, especially insoluble proteins including silk fibroin, elastin and keratin, acted as inducers for the production of a PLA-degrading

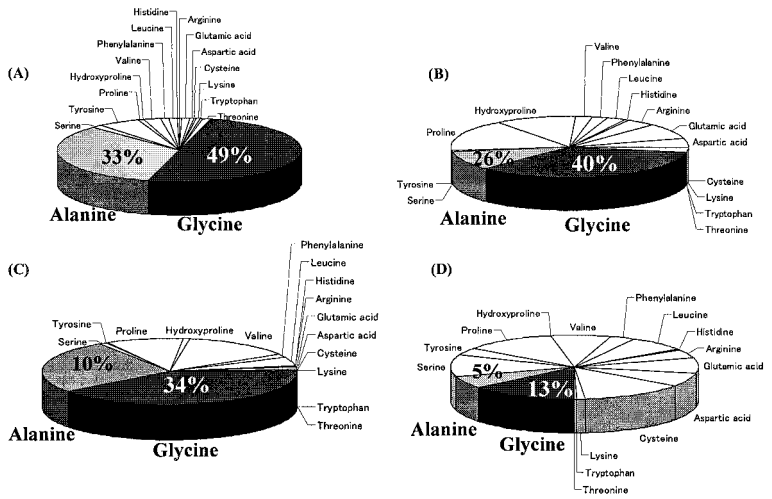

Figure 3. Amino acid compositions (mol%) of silk fibroin (A), gelatin (B), elastin (C) and keratin (D).

enzyme. Silk fibroin is an effective culture substrate for inducing enzyme production by *A. orientalis*. Some of the soluble peptides and amino acids also induce the enzyme production, but not as much as insoluble proteins. The culture broth without these inducers (control) had no enzymatic activity. The culture broths of *A. orientalis*, *S. waywayandensis* and *T. album* also showed protease activity against succinyl-(L-alanyl-L-alanyl-L-alanine)-

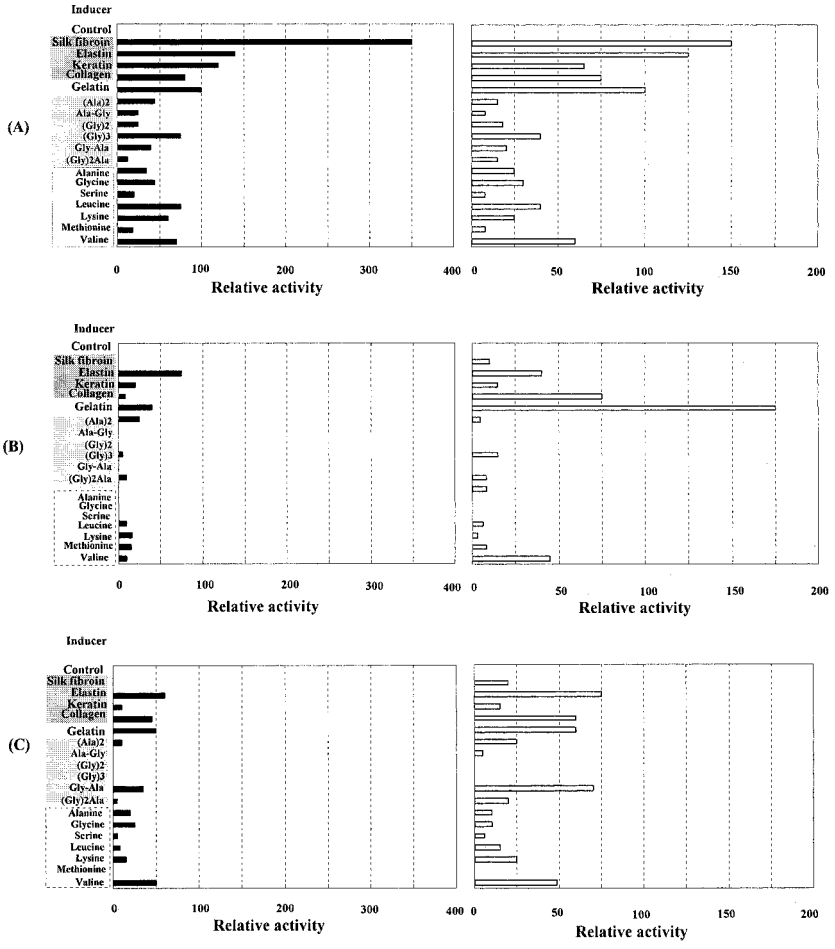

Figure 4. Effect of proteins, peptides and amino acids on PLA-degrading activity (■) and on Suc-$(Ala)_3$-*p*NA-degrading activity(□) of the culture broths obtained from *A. orientalis* (A), *S. waywayandensis* (B) and *T. album* (C). Activity of the enzyme from the culture broth of *A. orientalis* with gelatin was defined as 100% relative activity.

p-nitroanilide (Suc-$(Ala)_3$-*p*NA), a specific substrate for proteinase-K. Induction research is one of the biological tools/techniques for studying enzymes. The production of an enzyme took place when the strains recognized the substrates (inducers) added into the culture medium, then secreted the enzyme capable of degrading these substrates. It is interesting that different inducers may induce production of different types of enzymes.

Conclusions and Prospects

Although high molecular weight PLA film is not easily attacked by microorganisms, our studies showed that the degradation rate of PLA is markedly increased by the addition of gelatin into the culture medium. We also found that other natural substrates, including proteins, peptides and amino acids, acted as inducers for the production of PLA-degrading enzyme(s), and consequently accerelate the biodegradation of PLA.

In a number of recent studies, various prokaryotic microbes capable of degrading PLA have been reported. Nevertheless, *T. album* is the only eukaryotic microorganism capable of degrading PLA reported to date. The culture broth of this fungus also showed the enzymatic activity on PLA powder and other insoluble substrates, including silk fibroin powder and elastin. From the viewpoint of ecological impact, it is very important to study the biodegradation of PLA, especially by the enzymes of eukaryotes, including animal enzymes. Biological functions of these enzymes can be further extended to other new applications.

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