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Fundamental studies on enhanced degradation of plastics in landfill sites using biological functions

(微生物機能を利用した廃棄物埋立処分場におけるプラスチックの分解促進に関する基礎的研究)

A Thesis
Submitted to the Graduate School of Engineering
at
Osaka University

by
Tomonori Ishigaki

in a fulfillment of the requirements for
the degree
of Doctor of Philosophy
in Engineering

2000

Dedicated to
my parents and Mari
for their love

I am truly grateful
for their constant support
and encouragement

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Tomonori Ishigaki

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Chapter 1
General Introduction

The amount of municipal solid waste annually generated in Japan is estimated to 51 million tons and approximately 25 % of the waste are directly disposed into landfill sites (Japan Environmental Agency 1999). There have been occurring many problems with the waste disposal as society developed. Difficulty of acquiring the landfill sites is one of the concerning problems especially in the urban areas, whereas integrated solid waste management such as recycling or waste-to-energy has been developed. Therefore lack of waste landfill spaces has become a great public concern.

In particular, plastics are estimated to comprise up to 20 - 30 % of volume of the municipal solid waste in landfill (Franklin Associates Ltd. 1990). Since they are completely recalcitrant to microbial degradation, they semi-permanently remain there forms, and cause uneven settlement of the landfill sites. Further, they cannot be treated by environmentally friendly biological decomposition processes such as composting or anaerobic digestion. Thus plastic wastes are one of the most troublesome category and disposal of plastic waste has been blamed for shortening the life expectancy of landfill site.

The development of biodegradable plastics has been proposed as a solution for the problems of plastic wastes. Many biodegradable plastics have been developed in efforts to make materials more susceptible to biological degradation. Fig. 1.1 illustrates the general concept of the biodegradable plastic. The great merit of the plastics is an environmental suitability: theoretically, they are designed to be easily destroyed and finally to disappear under the natural environmental conditions including landfill sites or waste composting processes. As the plastics are made of natural materials and not of petroleum, they are recognized as new concept recycle products called Biorecycle or Ecorecycle products.

Biodegradable plastics in commercial level are divided into the following three groups depending on their main components (Table 1.1): bacterial polymers, polymers derived from plants or animals, and chemically synthesized polymers (Kawai 1995). A lot of research groups have focused on these materials and their properties as well as on possible useful applications.

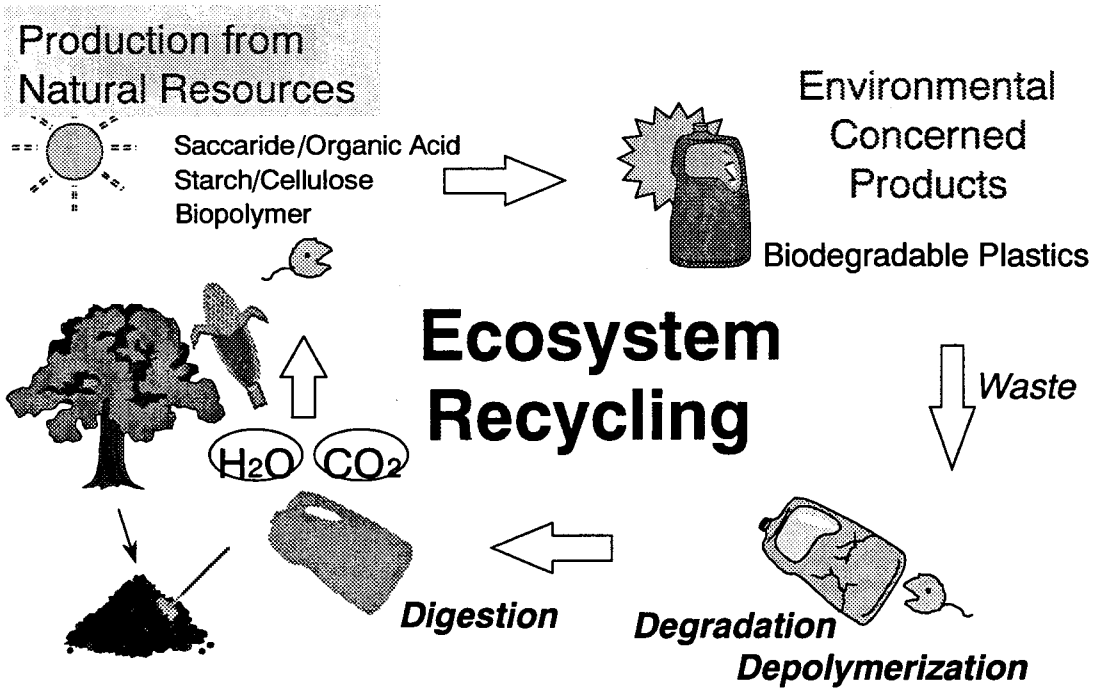

Fig. 1.1. General concepts of biodegradable plastic

However only a few investigations have focused on the fate of the plastics in waste disposal and treatment processes, and degradation mechanisms in detail have not been known yet. Biodegradability of the plastic in landfill site should highly depend on the biotic and abiotic factors at the given site. Consequently, the biodegradation processes of the plastics in landfills are not well characterized.

Table 1. 1. Several biodegradable plastics which have been already commercialized

Category	Characteristcs	Products (Company)	Yen/kg
Bacterial polymer			
Polyhydroxy alkanoate	good degradability	Biopol (Monsanto)	2-3000
	expensive	Biogreen (Mitsubishi Gas)	
Chemical synthesized polymers			
Poly-ε-caplolactone	low melting point	Celgreen (Daicel)	1200
	blendability with other resin	Tone (Union Carbide)	1000
		Biomicron (JSP)	
Polylactate	hard, high melting pint	Lacea (Mitsui Toatsu)	
	transparency	Lacty (Shimadzu)	2000
		Ecopla (Cargill)	1500
Polybutyrene succinate	flexible, strong	Bionolle (Showa Highpolymer)	900
	water-proofing	Skygreen (Sunkyong)	
Plant derived polymers			
Starch	hydrophilic	Mater-bi (Novamont)	800
		Novon (Warner Wranbert)	400-850
		Eco-Star (Saint Rolense)	850
		Ecoform (National Starch Chem)	
		Evercorn (Japan Corn Starch)	
Cellulose		Celgreen (Daicel)	
		Lunale (Planet Polymer)	
		Amipol (Michigan Biotech)	

In the most of the landfill sites, anaerobic condition generally dominates. Because of the slow rates of anaerobic biodegradation, the overall degradation of wastes occurring in landfill sites is slow. Recent studies have shown that food waste and newspapers excavated from landfills remains original forms after 20 years of burial (Franklin Associates Ltd. 1990). And there are many problems on anaerobic intermediates in leachate, and production of toxic, malodorous and explosive landfill gas. Considering such slow rates of biodegradation typical of landfills, the questions about degradability of the plastics in the waste landfill sites and their contribution to effective waste stabilization are still remaining.

The purpose of this study is enhancement of degradation of the plastic in the waste disposal and treatment processes as countermeasures against the landfill crisis. The schematic flow of this study is shown in Fig. 1.2. In Chapter 2, the population sizes of polymers-degrading microorganisms in a landfill site were estimated in order to assess the *in situ* plastic degradation

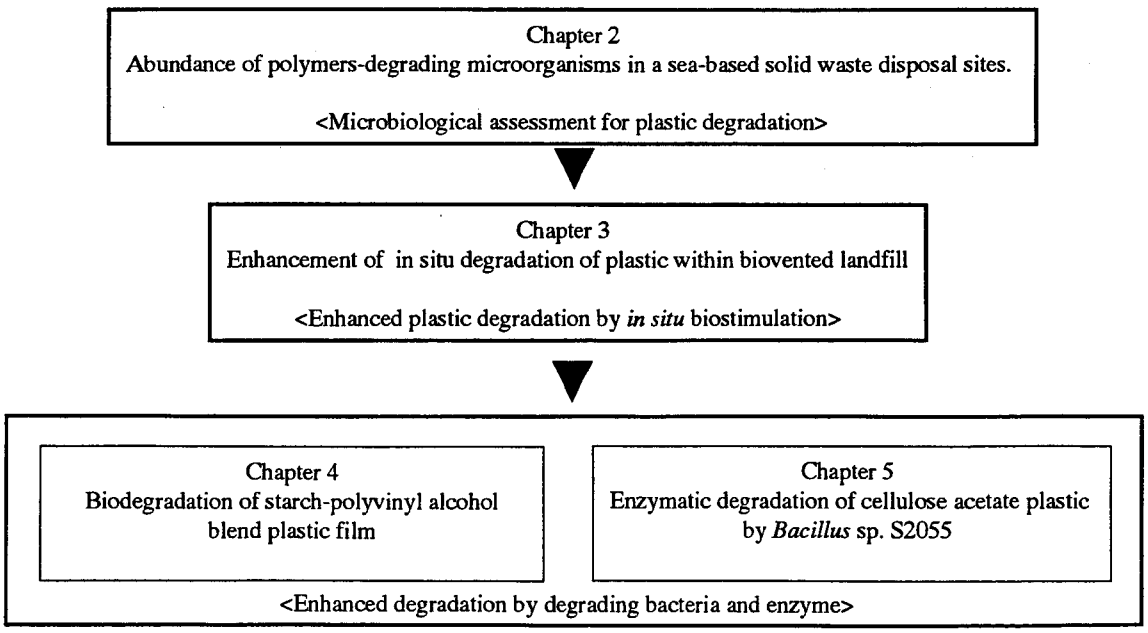

Fig. 1.2. Schematic flow of this study

potentials. The five kinds of biodegradable polymers as plastic-constituting materials, poly-β-hydroxybutyrate, poly-ε-caprolactone, cellulose acetate, polylactic acid and polyethylene glycol, were subjected to the investigations. In Chapter 3, enhancement of *in situ* degradation of plastics using the biostimulation technique was studied regarding with engineered landfills designed by previous reports (Pohland *et al.* 1994, Townsend *et al.*1995, U.S. Environmental Protection Agency, 1991). The fate of commercial biodegradable plastic films, Biopol (heteropolymer of poly-β-hydroxybutyrate and valerate), Celgreen P-HB (poly-ε-caprolactone), Mater-bi (Starch–Polyvinyl alcohol blend) and Celgreen P-CA (cellulose acetate) in the model landfill reactor, were examined with and without biostimulation. In Chapters 4 and 5, in order to promote the plastic degradation further, specific degrading microbes and enzymes were applied to plastics which were not effectively degraded even in the engineered landfill reactor. Chapter 4 deals with the degradation of Mater-bi using the PVA-degrading bacterium and enzyme. In Chapter 5, CA-degrading bacterium was isolated, and the degrading mechanisms of CA were investigated in detail. Based on the results, feasibility of bioaugmentation or enzymatic pretreatment was discussed.

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Chapter 2

Abundance of Polymers Degrading Microorganisms in a Sea-Based Solid Waste Disposal Site

2.1. INTRODUCTION

Biodegradability of plastics under *in situ* conditions, especially in the landfill sites, has been poorly or scarcely understood until now. Therefore, anyone cannot answer the question "would biodegradable plastics be actually degraded in the landfill sites and, accordingly, can they efficiently contribute to the waste reduction ? " Thus, it is necessary to know more about the plastic degradation potentials in the landfill sites. From recent works, it has been concluded that microorganisms capable of degrading polymer components might play a very important role in destruction of plastics (Schirmer *et al.*, 1993, Ishigaki *et al.*, 1999). This encouraged us to investigate the abundance of polymer-degrading microorganisms (PDMs) in a selected landfill site.

In this chapter, the distribution of PDMs in a sea-based, municipal solid waste landfill site in Japan was investigated. Both aerobic and anaerobic PDMs capable of degrading selected plastic-constituting materials, i. e. poly-β-hydroxybutyrate (PHB), poly-ε-caprolactone (PCL), cellulose acetate (CA), polylactic acid (PLA) and polyethylene glycol (PEG), were enumerated. Heterotrophic bacterial flora in the same samples was also investigated, as they seem important as the background microbes of the PDMs.

2.2. MATERIALS AND METHODS

2.2.1. Landfill site and sample collection

The leachate samples studied in this work were taken from the south section of Osaka North Port sea-based solid waste disposal site (Furukawa *et al.*, 1994). Fig. 2.1 shows the location of the landfill site. The landfill consists of three sites, and only Site I is being used for disposal of solid wastes. The area of Site I is 73 ha and consists of the facultative pretreatment pond (FPP) and the aerated lagoon (AL). The FPP is a common sea-based landfill site, being directly disposed of the wastes. On the other hand, the AL is mechanically aerated for reducing organic pollutants of the leachate from the FPP before being discharged into the open sea via Site II and III. The leachate samples were collected seasonally (4 times) from both sampling point No. 1 (FPP) and No.2 (AL) between April 1997 to January 1998.

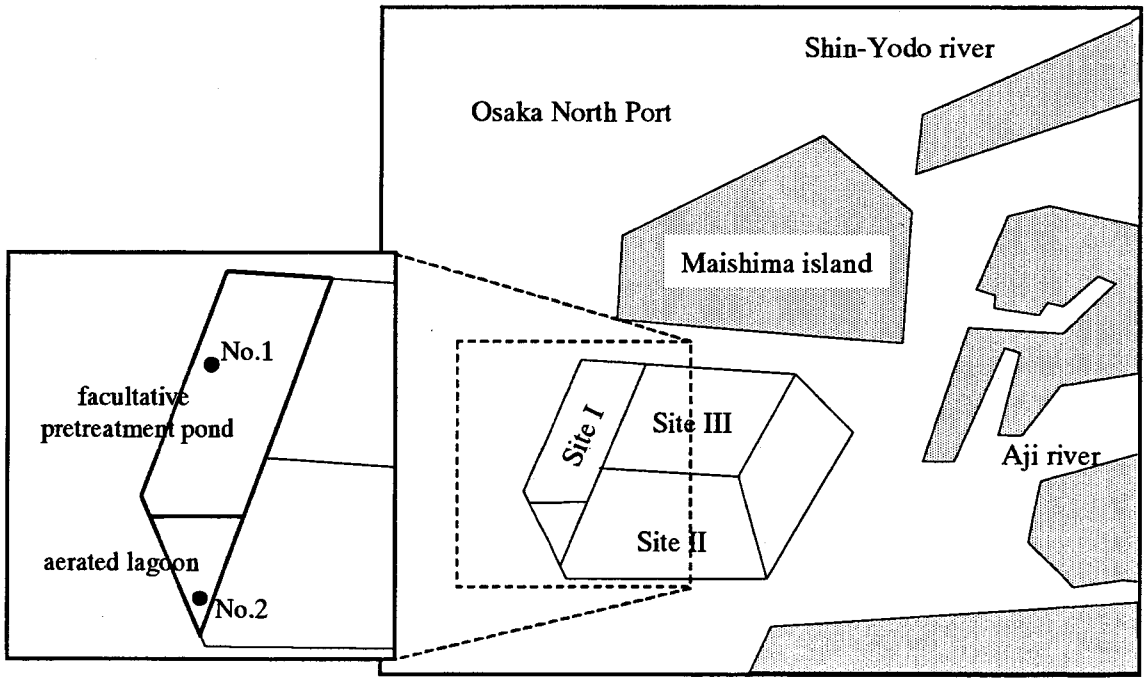

Fig. 2.1. Location of Osaka North Port sea-based waste disposal site.

2.2.2. Polymers

PHB (Aldrich Chemical Company Inc., molecular weight (Mw) average = 420000), PCL (Wako Pure Chemical Co. Ltd., Mw av. = 10000), CA (Acros Organics, Mw av. = 100000), PLA (Wako Pure Chemical Co. Ltd., Mw av. = 5000) and PEG (Kishida Chemical Co. Ltd., Mw av. = 4000) were used as plastic-constituting polymers. PHB is a polymer based on bacterially produced polyester. It is a bacterial storage material. CA is an acetyl substitute of cellulose, which is produced as a renewable resource from plant. These polymers were categorized to natural polymers (Demicheli 1996). On the other hand, PCL, PLA and PEG are made of petroleum-based polyester or polyether. Thus these polymers were categorized to chemically synthesized polymer.

2.2.3. Enumeration of PDMs

Agar plates containing the emulsified polymers were prepared by procedure described below according to Morikawa *et al.* (1978) and Augusta *et al.* (1993). 1000 mg of each polymer was dissolved in 20 ml of methylene chloride. Each solution was emulsified with a homogenizer (TOMY Seiko Co. Ltd., Tokyo, Japan, UD-210, 10 min x 3) into 1000 ml of a basal medium (K_2HPO_4: 1000 mg/l, $(NH_4)_2SO_4$: 1000 mg/l, $MgSO_4 \cdot 7H_2O$: 200 mg/l, $FeCl_3$: 10 mg/l, NaCl: 50 mg/l, $CaCl_2$: 50 mg/l, yeast extract (Difco): 250mg/l) containing 100 mg/l of TritonX-100. The medium was made using artificial seawater (NaCl: 30 g/l, $MgCl_2 \cdot 6H_2O$: 10.8 g/l, $MgSO_4$: 5.4 g/l, KCl: 700 mg/l, $CaCl_2 \cdot 6H_2O$: 1.0 mg/l, $FeSO_4 \cdot 7H_2O$: 1.0 mg/l, $MnSO_4 \cdot 4H_2O$: 1.0 mg/l). Agar (1.5 %, w/v) was added to the emulsified medium and dissolved by heating, which simultaneously evaporated methylene chloride from the medium. Enumeration of the PDMs was performed using these emulsified polymer media by the plate count technique. The leachate samples were plated onto the media after appropriate dilution and they were cultivated at 28°C

for 10 days, according to Nishida *et al* (1993). Resultant colonies with halos, which indicate polymer degradation, in the medium, were counted as PDMs. Such a halo formation would suggest the depolymeraization of polymer in the agar-plate (Nishida and Tokiwa 1993). For enumerating aerobic and anaerobic PDMs, the plates were cultivated under ambient atmosphere and in an anaerobic incubator (Tabai Espec Co. Ltd., Osaka, Japan, EAN-140; N_2 : CO_2 : H_2 = 90 : 5 : 5), respectively.

2.2.4. Enumeration, isolation and taxonomical classification of heterotrophic bacteria

1/4 PYG agar medium (bacto-peptone (Difco): 500 mg/l, yeast extract (Difco): 250 mg/l, glucose: 125 mg/l) was used for enumerating heterotrophic bacteria. The medium was prepared using the above-mentioned artificial seawater. The appropriately-diluted leachate samples were plated onto 3-5 plates and they were incubated at 28 °C for 5 days under either aerobic or anaerobic conditions. Colonies formed on the plates were counted as the aerobic and anaerobic heterotrophic bacteria, respectively. Almost all colonies formed on the plates were picked up, subcultured twice in 1/4 PYG liquid medium, and isolated as the pure cultures of representative bacteria present in the landfill leachate. The aerobic isolates were morphologically and physiologically characterized and identified up to genus level according to Cowan and Steel (1973) and Bergey's Manual (1984). Bacterial identification kits (API20NE and/or API20E; Bio Merieux S.A.) were also used for ensuring the identification. The anaerobic heterotrophic isolates were also morphologically and physiologically characterized and identified up to genus level according to Anaerobe Laboratory Manual (Holdeman *et al.*, 1977), based on the results of analyses of acetate, propionate, butyrate and lactate accumulated in the culture broth. The organic acids in the culture broth were analyzed using a Hitachi 263-50 type gas chromatography equipped with a FID with Thermon 3000 column.

2.2.5. Diversity index of bacterial flora

Simpson Index (Simpson, 1949) expressed by the following equation was used to characterize the diversity of the heterotrophic bacterial community in the leachate samples.

$$\beta = \frac{N(N-1)}{\sum_i n_i(n_i-1)}$$

N: the number of total heterotrophs isolated, n_i: the number of isolates of each genus or category.

2.3. RESULTS

2.3.1. Physico-chemical conditions of the landfill site

Physico-chemical conditions of the two sampling points of the landfill site, FPP and AL, are summarized in Tables 2.1 and 2.2, respectively. The indices investigated here showed similar values for both sampling points throughout the experimental period except for dissolved oxygen (DO) concentration. The DO values in the FPP and AL were 1.4-1.6 and 3.9-9.4 mg/l, respectively, and seem to be maintained at relatively high levels as a sea-based landfill site (Japan Society of Civil Engineering, 1998). The water temperature varied from 5.5 - 6.0 °C in winter to 28.0 - 28.5 °C in summer. The concentrations of organic matter varied little within the ranges around 5-10 mg/l as BOD (biochemical oxygen demand) and 35-50 mg/l as COD (chemical oxygen demand), which were similar ranges observed in other sea-based landfill sites (Japan Society of Civil Engineering, 1998).

Table 2.1. Physico-chemical conditions at sampling point No. 1 (FPP).

	Spring (4/23/1997)	Summer (9/11/1997)	Autumn (11/7/1997)	Winter (1/14/1998)
Water temp. (°C)	14.2	28.5	17.5	6.0
PH	7.9	8.1	7.8	8.2
DO (mg/l)	1.5	1.4	1.6	1.4
BOD (mg/l)	12.4	5.6	5.0	5.6
COD (mg/l)	50.0	37.2	34.4	36.1

Table 2.2. Physico-chemical conditions at sampling point No. 2 (AL).

	Spring (4/23/1997)	Summer (9/11/1997)	Autumn (11/7/1997)	Winter (1/14/1998)
Water temp. (°C)	14.0	28.0	17.0	5.5
pH	7.9	8.2	7.9	8.2
DO (mg/l)	4.1	3.9	4.7	9.4
BOD(mg/l)	12.5	9.1	7.2	5.3
COD(mg/l)	52.0	35.6	33.5	36.4

2.3.2. Distribution of PDMs

Results of the enumeration of PDMs in the leachate samples from the FPP and AL are summarized in Tables 2.3 and 2.4, respectively, in comparison with the heterotrophic bacterial counts. Both aerobic and anaerobic PDMs for 5 kinds of polymers were detected from almost all samples. Especially, CA- and PHB-degraders were found from all the samples, and accounted for more than 0.1 % of the heterotrophic populations in most cases. On the other hand, the spring sample from the FPP did not contain detectable numbers of aerobic PLA- and PEG- and anaerobic PEG-degraders, and the winter sample from the AL did not anaerobic PCL- and PEG-degraders. Further, the ratios of PCL-, PLA- and PEG-degraders were often much lower than 0.1 %, even when they were detected.

The population sizes of the PDMs seasonally fluctuated both in the FPP and AL samples considerably. The counts of aerobic PDMs tended to be higher than those of anaerobic PDMs in the AL samples, however, no such a tendency was observed in the FPP samples. Fig. 2.2 shows a typical example of the appearance of colonies on the PDM-counting media (the emulsified polymer media) after 10-day incubation. The halo formation by the aerobic and anaerobic PDMs is compared in the figure. The halo sizes, which seem to represent the degradation activity (Morikawa *et al.*, 1978), formed by the aerobic PDMs are much larger than those formed by the anaerobic PDMs in general.

Table 2.3. Results of the enumeration of heterotrophic bacteria and PDMs in the FPP samples (CFU/ml). Ratios of the PDMs population to the heterotrophic bacteria were also shown.

		heterotroph	polymer degrader				
			CA	PHB	PCL	PLA	PEG
Spring	anaerobe	3.3×10^4	9.0×10^2	2.8×10^3	5.0×10^3	7.5×10^3	N.D.
			2.7%	8.5%	15%	23%	–
	aerobe	1.0×10^5	1.0×10^2	3.8×10^3	2.0×10^2	N.D.	N.D.
			0.1%	3.8%	0.2%	–	–
Summer	anaerobe	1.4×10^6	1.6×10^4	2.3×10^4	1.0×10^4	2.8×10^4	1.2×10^4
			1.1%	1.6%	0.7%	2.0%	0.9%
	aerobe	1.5×10^5	2.7×10^4	2.5×10^4	3.2×10^4	3.9×10^4	3.3×10^4
			18%	17%	21%	26%	22%
Autumn	anaerobe	8.8×10^5	7.0×10^4	2.5×10^5	5.8×10^4	8.0×10^2	2.4×10^4
			8.0%	28%	6.6%	0.09%	2.7%
	aerobe	6.5×10^4	6.7×10^1	2.0×10^2	6.6×10^1	6.6×10^1	3.3×10^1
			0.1%	0.3%	0.1%	0.1%	0.05%
Winter	anaerobe	2.1×10^5	2.7×10^2	7.9×10^2	2.3×10^2	6.2×10^2	1.0×10^1
			0.1%	0.4%	0.1%	0.3%	0.005%
	aerobe	8.8×10^4	3.5×10^2	4.7×10^3	1.5×10^2	1.1×10^1	7.4×10^2
			0.4%	5.3%	0.2%	0.01%	0.8%

N.D.: not detected (less than 1 CFU/ ml)

Table 2.4. Results of the enumeration of heterotrophic bacteria and PDMs in the AL samples (CFU/ml). Ratios of the PDMs population to the heterotrophic bacteria were also shown.

		heterotroph	polymer degrader				
			CA	PHB	PCL	PLA	PEG
Spring	anaerobe	1.3×10^{5}	4.0×10^{2}	3.4×10^{4}	3.3×10^{0}	6.0×10^{2}	1.0×10^{2}
			0.3%	26%	0.003%	0.5%	0.08%
	aerobe	5.4×10^{5}	1.0×10^{4}	7.4×10^{3}	8.0×10^{2}	1.0×10^{3}	1.0×10^{3}
			1.9%	1.4%	0.2%	0.2%	0.2%
Summer	anaerobe	1.4×10^{6}	3.5×10^{3}	6.1×10^{3}	2.4×10^{3}	8.0×10^{2}	9.0×10^{3}
			0.3%	0.4%	0.2%	0.06%	0.6%
	aerobe	5.8×10^{5}	4.1×10^{4}	1.8×10^{4}	5.6×10^{4}	4.1×10^{4}	2.8×10^{4}
			7.1%	3.1%	9.7%	7.1%	4.8%
Autumn	anaerobe	2.1×10^{4}	1.0×10^{2}	8.0×10^{3}	1.0×10^{2}	8.0×10^{2}	2.3×10^{2}
			0.5%	38%	0.5%	3.8%	1.1%
	aerobe	5.6×10^{4}	1.1×10^{2}	1.5×10^{2}	1.2×10^{2}	2.0×10^{2}	1.5×10^{1}
			0.2%	0.3%	0.2%	0.4%	0.03%
Winter	anaerobe	3.4×10^{4}	2.0×10^{1}	1.0×10^{1}	N.D.	2.0×10^{1}	N.D.
			0.06%	0.03%	–	0.06%	–
	aerobe	4.7×10^{4}	4.8×10^{1}	1.1×10^{2}	3.1×10^{2}	2.9×10^{2}	2.5×10^{2}
			0.1%	0.2%	0.7%	0.6%	0.5%

N.D.: not detected (less than 1 CFU/ ml)

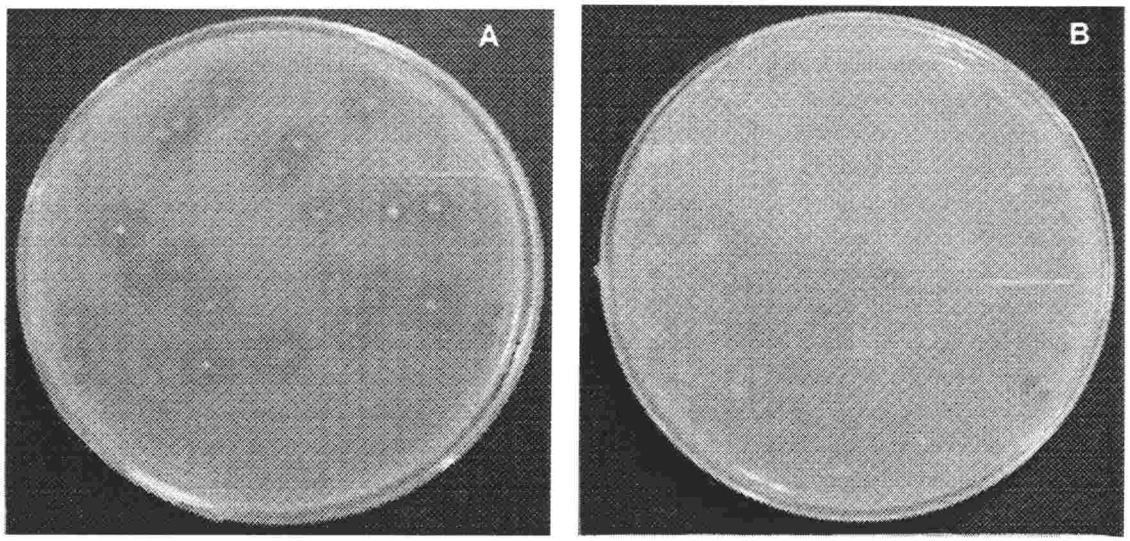

Fig. 2.2. A typical example of appearances of the colonies of (A) aerobic and (B) anaerobic PDMs. Figures shows the colonies and halos on the PCL emulsified plates.

2.3.3. Heterotrophic bacterial flora

Data on the heterotrophic bacterial abundance in the FPP and AL samples are given in Tables 2.3 and 2.4, respectively. The aerobic heterotrophic bacterial counts ranged from 4.8 x 10^4 to 5.8 x 10^5 CFU/ml, while the anaerobic heterotrophs from 2.1 x 10^4 to 1.4 x 10^6 CFU/ml. The dominant aerobic and anaerobic bacterial genera in the leachate samples are expressed as the ratios of bacterial genera or categories (e.g., Coryneforms) as shown in Figs. 2.3 and 2.4, respectively. Both the aerobic and anaerobic bacterial flora varied seasonally and between in the FPP and AL samples. The aerobic bacterial genera or categories commonly isolated from the FPP and AL samples were *Acinetobacter*, *Pseudomonas*, *Yersinia*, *Bacillus* and Coryneforms, while the anaerobic bacteria were *Eubacterium*, *Fusobacterium*, *Propionibacterium* and *Veillonella*. According to the calculation of Simpson index defined in the previous part, diversity of aerobic bacterial flora was higher in the AL samples than in the FPP samples as a whole. On the other hand, the contrary trend was observed on the diversity of the anaerobic bacterial flora.

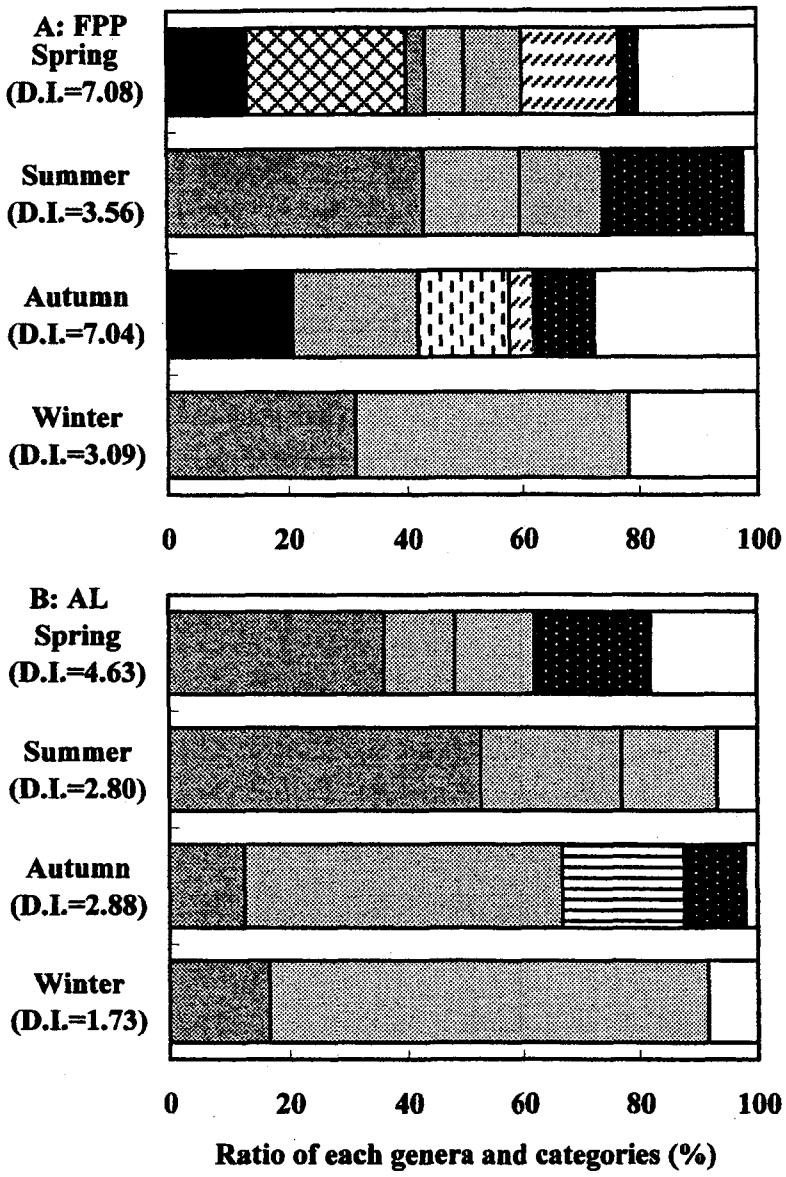

Fig. 2.3 Anaerobic bacterial flora in (A) FPP and (B) AL

Eubacterium *Bacteroides* *Fusobacterium*
Propionibacterium *Butyrivibrio* *Leptotrichia*
Peptostreptococcus *Streptococcus* *Veillonella*
other

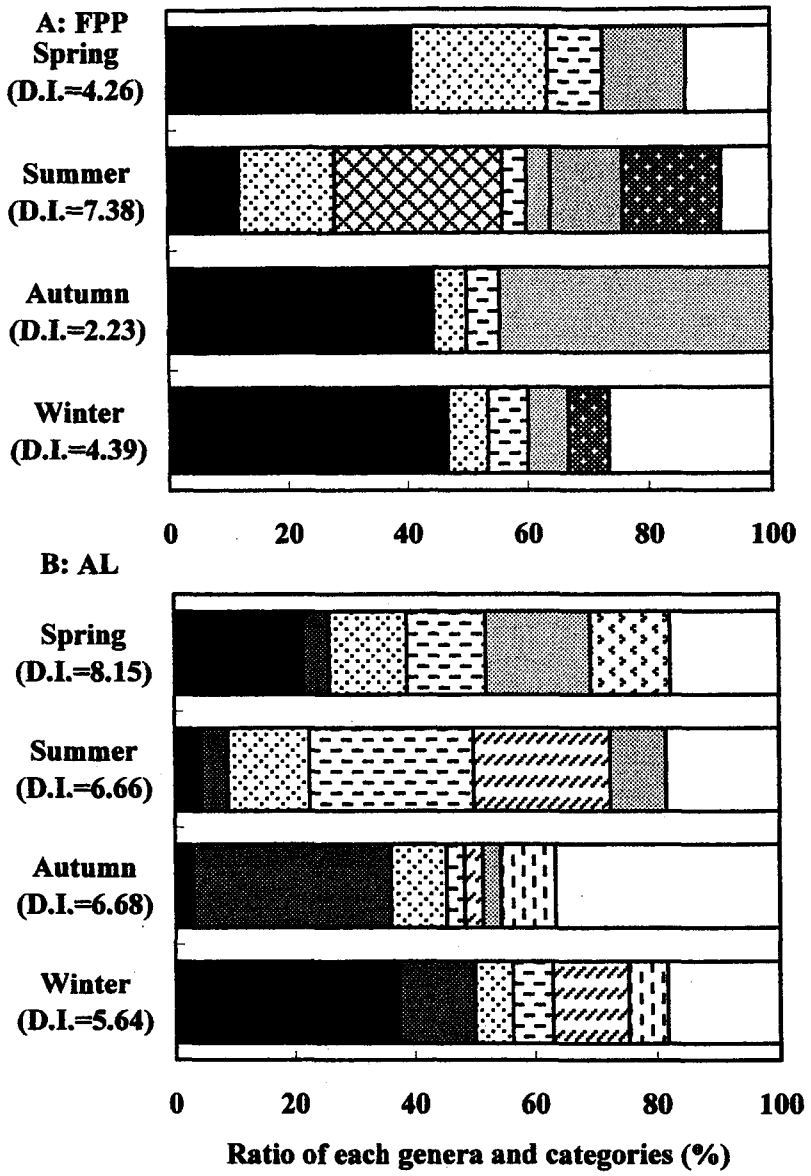

Fig. 2.4 Aerobic bacterial flora of (A) FPP and (B) AL

■ Coryne form ■ *Haemophilis* ■ *Acinetobacter* ■ *Pseudomona*
■ *Alcaligenes* ■ *Acromobactor* ■ *Bacillus* ■ *Staphylococ*
■ *Veillonella* ■ *Yersinia* ■ *Chryseomonas* ■ other

2.3.4. Correlation between the population sizes of PDMs and heterotrophic bacteria

Fig. 2.5 shows the relationship between the counts of PDMs and heterotrophic bacteria. As shown in the figure, the population of PDMs had a significant correlation with that of heterotrophs (correlation coefficient; R = 0.68). A higher correlation (R = 0.67) was observed between the counts of aerobic PDMs and heterotrophs than between anaerobic ones (R = 0.53). Although relationships between population sizes of the PDMs and other parameters, e.g., water temperature, DO, BOD, COD, diversity of heterotrophic bacterial flora etc., were also investigated, no significant correlation was found (data analyses not shown).

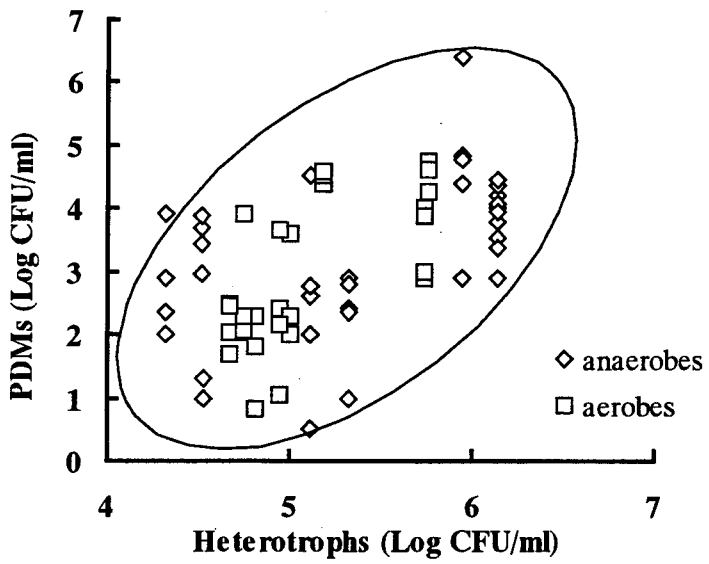

Fig. 2.5. Relationship between population sizes of the PDMs and heterotrophic bacteria

2.4. DISCUSSION

Although the information on the abundance and/or distribution of PDMs in the natural environment is quite important for assessing the bigdegradability of plastics, it has been dealt with by only a few studies up to date (Potts *et al.*, 1972, Nishida *et al.* 1994). Especially

concerning the PDMs in the landfill sites, there has been only one paper describing the presence of aerobic PCL- and PHB-degraders in two landfill leachate samples from Tokyo Bay, Japan (Nishida *et al.*, 1994). Here we have extensively investigated the distribution of PDMs in the leachate from the sea-based landfill site for five kinds of plastic-constituting polymers.

PDMs for each polymer were detected at certain high levels from almost all samples, indicating ubiquitous and abundant presence of PDMs for various polymers in the landfill sites. In other words, plastic degradation potentials are commonly present in the landfill sites. Although PDMs for natural polymers, PHB and CA were found from all the samples, those of chemically-synthesized polymers, PCL, PLA and PEG could not be always detected. Further, PDMs for natural polymers accounted for higher percentages to the heterotrophic bacteria than PDMs for chemically-synthesized polymers as a whole. Torres *et al.* (1996) also reported that only a few kinds of microorganisms could degrade PLA- and LA-containing polymers, and Kawai (1989) reported that higher-molecular-weight PEG showed very low biodegradability. This suggests that the degradation potential of plastics composed of chemically-synthesized polymers in the natural environments including landfill sites is inferior compared to that of natural polymers.

In this study, the leachate samples collected from the FPP and AL were used for enumerating PDMs. In both locations, similar environmental conditions were observed except for the DO concentration. In the AL, where mechanical aeration created a very high DO level, populations of the aerobic PDMs and heterotrophs tended to be larger than the anaerobic ones, and the diversity of the aerobic heterotrophs was higher than in the FPP. Further, judging from the bacterial genera and/or categories shown in Fig. 2.3, percentages of the strict aerobes to the

total aerobic heterotrophs were higher than in the FPP. These indicate that selective growth of aerobic bacteria including PDMs occurred in the AL. On the other hand, relative abundance and diversity of anaerobic PDMs and heterotrophs in the FPP were higher than in the AL, though the DO concentration in the FPP was maintained at relatively high levels. Since the aerobic PDMs possessed higher polymer degrading activities compared with the anaerobic PDMs judging from the halo sizes (Fig. 2.2), activation of aerobic PDMs by the mechanical aeration of leachate might be an effective mean to accelerate the plastic degradation in the landfill sites.

The aerobic and anaerobic heterotrophic bacterial flora were also investigated as the background microbes of the PDMs, and a relatively high correlation between populations of PDMs and heterotrophs was shown, suggesting that the PDMs play a certain role in the microbial community. We reported here the dominant bacterial genera and/or categories and frequent transition of bacterial flora in landfill site. Some of the dominant aerobic heterotrophic bacteria found in this study were also reported by Kawai *et al.* (1988). However, still little has been known, and further intensive studies are necessary to clarify the microbial ecology of the landfill sites.

From the results of this study, it may be concluded that certain degradation potentials of various kinds of biodegradable plastics exist in the landfill sites, and that the proper management or operation of the environmental conditions, e.g. mechanical aeration of the leachate, will lead to stimulation of PDMs. Strategies of the management or operation of the landfill sites as the bioreactors utilizing indigenous microbes have been already proposed by Pohland *et al.* (1995) and Barlaz *et al.* (1989). Several previous studies have been concluded that polymers-degrading microorganisms would play a very important role in destruction of

plastics (Schirmer *et al.*, 1993, Ishigaki *et al.*, 1999). Therefore, by applying the bioreactor landfill techniques, biodegradable plastics may show the enhanced degradability in waste landfill site.

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Chapter 3
Enhancement of Degradation of Plastics by Bioventing in Waste Landfills

3.1. INTRODUCTION

In Chapter 2, the abundance of PDMs in the sea-based solid waste landfill site was shown. The results indicated that certain degradation potentials for various kinds of biodegradable plastics exist in the landfill site, and that the proper management or operation of the environmental conditions will lead to stimulation of PDMs and, consequently, enhance decomposition of plastic wastes. Intentional aeration into a landfill site seems to be an effective mean for accelerating the plastic degradation. However researches on the *in situ* biodegradation of plastics and its enhancement in waste landfill sites have not been reported yet. The objective of this chapter is to clarify the biodegradation behavior of degradable plastics in the landfill sites using landfill model reactors. Degradabilities of commercial degradable plastics within the model landfill reactors with and without bioventing were investigated. Bioventing is the process of aerating soils to stimulate indigenous biological activity and promote the aerobic biodegradation. Nowadays it has been applied as one of cost-effective technologies for bioremediation of contaminated sites.

3.2. MATERIALS AND METHODS

3.2.1. Plastics

Commercially available biodegradable plastics were used for degradation test in waste landfill reactors. Biopol D411G, a polyester of polyhydroxybutyrate (92%)/ valerate (8%) (PHB/V; Monsanto Co.) and Celgreen HBT, a polyester of polycaprolactone (PCL; Daicel Chemical Co.) were selected as typical microbially and chemically derived plastics, respectively. Celgreen PCA, cellulose acetate (CA; Daicel) and Mater-bi AF10H, starch (60%)/ polyvinyl alcohol (40%) blend (starch-PVA; The Nippon Synthetic Chemical Ind.) were selected as typical natural homopolymer and blend of natural and synthetic polymers, respectively.

3.2.2. Degradation of plastic within model landfill reactors

A 300-ml Erlenmeyer flask was filled with synthesized waste (Ishigaki *et al.* 1999) and it was compacted to a density of 430 kg-wet waste/m^3. The flask was equipped with air injection tube system at the center of the bottom. A traditional waste landfill was simulated by the flask, without aeration (TL reactor), while a landfill applying bioventing was simulated by the flask with 500mL/min of aeration (biovented landfill: BVL). Bioventing is the process of appropriately venting and/or sucking the vadose zones to stimulate indigenous biological activity and promote the aerobic biodegradation. Nowadays it has been applied as one of cost-effective technologies for bioremediation of contaminated sites. In BVL reactor, water addition at intervals was coupled with aeration, to maintain the water contents of the synthetic waste at about 50%. One plastic film (50 x 50 mm) was placed in the middle of the reactors.

3.2.3. Determination of weight and thickness of plastic film

The plastic film was taken from the reactor, washed with deionized water, dried for 30

min in a desiccater, and its wet weight was measured. When the film was broken into small pieces, they were collected on a 24-mesh sieve for measuring the weight. In preliminary tests, the proportionality between the wet weight (measured by this method) and dry weight of the plastic film in the degradation process was confirmed. Film thickness was measured by a micrometer (Mitsutoyo Co. Ltd., Tokyo)

3.2.4. Scanning electron microscopy (SEM) observation

Film samples were coated with gold by JFC-1100E (JEOL, Tokyo, Japan) and the surface was investigated with a JEOL 5410LV scanning electron microscope (JEOL).

3.2.5. Enumeration of polymer degrading microorganisms (PDMs)

PHB-, PCL- and CA-degrading microbes were enumerated by the procedure described in 2.2.3. PVA- or starch-degrading microbes were detected by PVA- or starch-containing BSM (K_2HPO_4: 1000 mg/l, $(NH_4)_2SO_4$: 1000 mg/l, $MgSO_4 \bullet 7H_2O$: 200 mg/l, $FeCl_3$: 10 mg/l, NaCl: 50 mg/l, $CaCl_2$: 50 mg/l, yeast extract (Difco): 250mg/l, starch or PVA 1 g/l). Enumeration of the PDMs was performed using the polymer containing media by the plate count technique. One gram of the waste sample was suspended to 99 ml of distilled water. After 30 minutes agitation, suspension was diluted and plated onto the media and they were cultivated at 28°C for 10 days, referred to Nishida *et al* (1993). Resultant colonies with halos, which indicate the polymer degradation in the medium, were counted as PDMs. PVA- and starch-degraders were detected by addition of Iodine solution onto media, and each colony with apparent halo as isolated and its PVA- or starch-degrading activity was confirmed by the liquid cultivation.

3.3. RESULTS

3.3.1. Degradation of PHB/V plastic

Fig. 3.1 shows the periodical changes in the weight loss and film thickness of PHB/V plastic in the TL and BVL reactors. In the TL reactor, PHB/V film did not show any significant weight loss during 120 days of burial, whereas the film thickness decreased from 54μm to 45μm. On the other hand, significant weight loss of PHB/V was observed in the BVL reactor. The weight loss proceeded linearly for 100days, and finally the film disappeared. The film thickness decreased to 39.3 μm in 25 days, and subsequent measurement was impossible because of the film breakage as shown in Fig. 3.2. From the SEM observation, the film surface was totally eroded and the smoothness was lost (Fig. 3.3). Bacterial population of PHB-degrader in the BVL increased 1-2 orders of magnitude compared to those in the TL (Fig. 3.4), indicated the stimulation of the bacterial population by the bioventing.

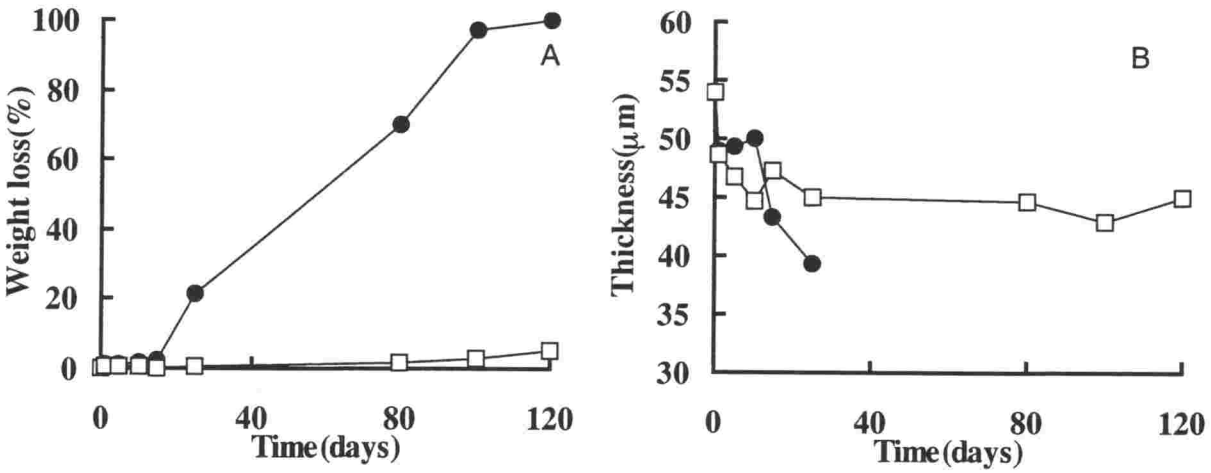

Fig. 3.1. Degradation of PHB/V plastic film in the TL (□) and BVL (●) reactors. A: weight loss of film, B: film thichness

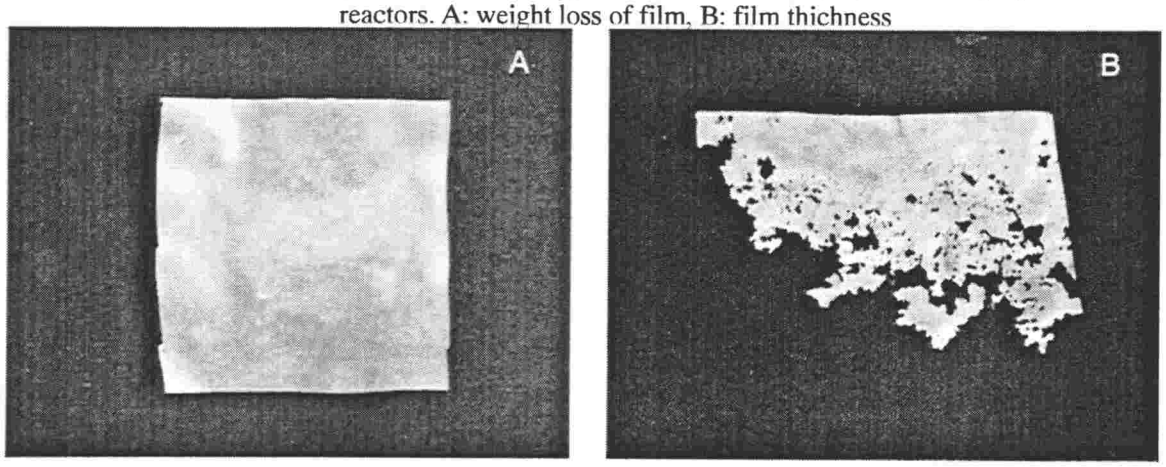

Fig. 3.2. PHB/V plastic film degraded in the reactors.
A: after 120-day degradation in TL, B: after 40-day degradation in BVL

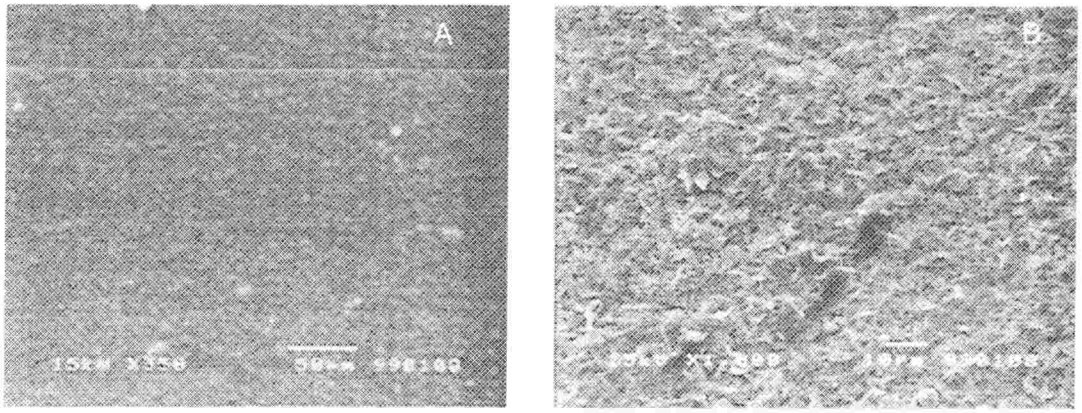

Fig. 3.3. SEM observation of the surface of PHB/V plastic films.
A: before the degradation, B: after the 80-day degradation in the BVL.

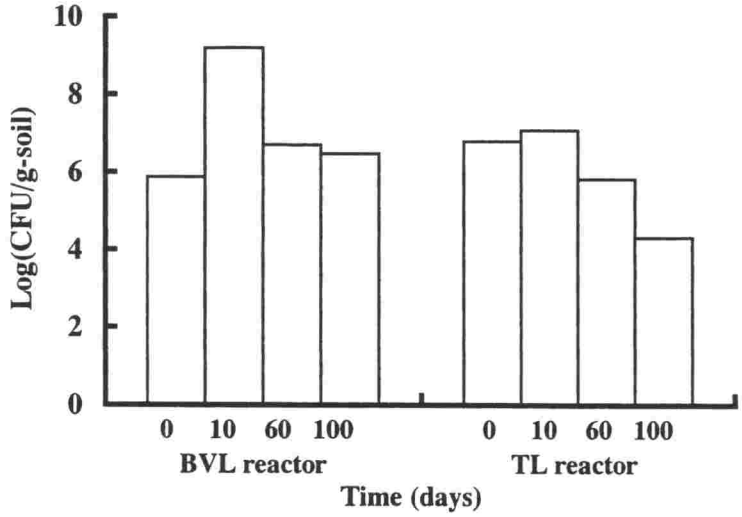

Fig. 3.4 Population of PHB degraders in the TL and BVL reactors.

3.3.2. Degradation of PCL plastic

Fig. 3.5 shows the changes in the weight loss and film thickness of PCL plastic in the TL and BVL reactors. In both reactors, the weight loss of PCL films showed a similar trend. The weight loss slowly proceeded in the initial 80 days, followed by the rapid degradation. The weight loss proceeded a little more rapidly in the TL than in the BVL reactor, and the final weight losses after 120 days reached 29.7 and 17.3 % in the TL and BVL reactors, respectively.

Slow decrease of film thickness was shown in the TL reactor, whereas any significant change was not observed in the BVL. Although biodegradation of the PCL plastic film was not enhanced by the bioventing, remarkable breakage of the film started from 40days and luminescence of its surface was lost (Fig. 3.6). Many pinholes on the film surface and partial degradation from eroded holes were observed (Fig. 3.7). Fig. 3.8 shows the population sizes of PCL-degrader in both reactors. The counts of PCL-degrader in the BVL were higher than those in the TL through the experimental period. The PCL-degrader, as well as the PHB-degrader, seemed to be activated by the bioventing.

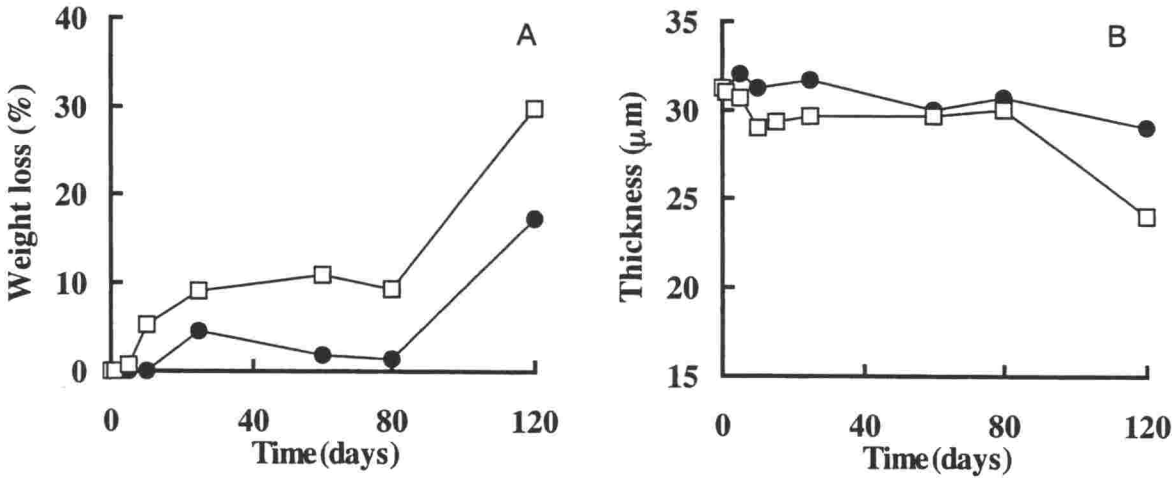

Fig. 3.5. Degradation of PCL plastic film in the TL (□) and BVL (●) reactors. A: weight loss of film, B: film thickness

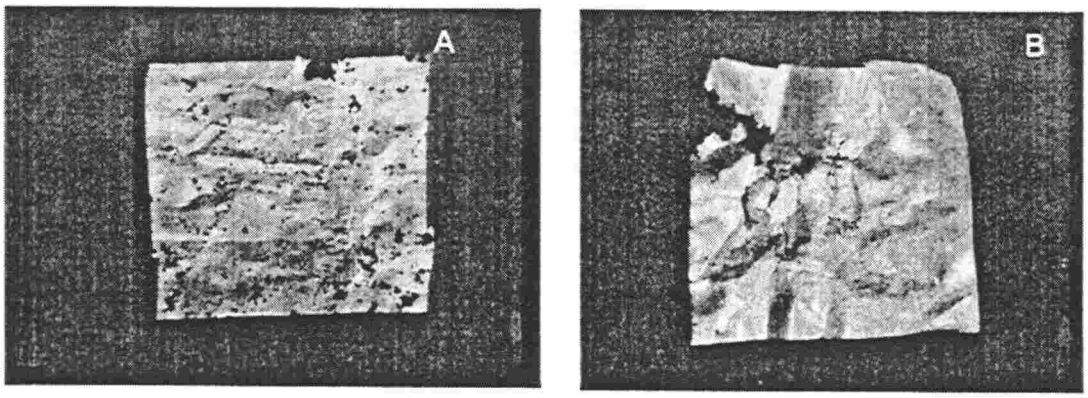

Fig. 3.6. PCL plastic film degraded in the reactors
A: after 120-day degradation in the TL, B: after 120-day degradation in the BVL

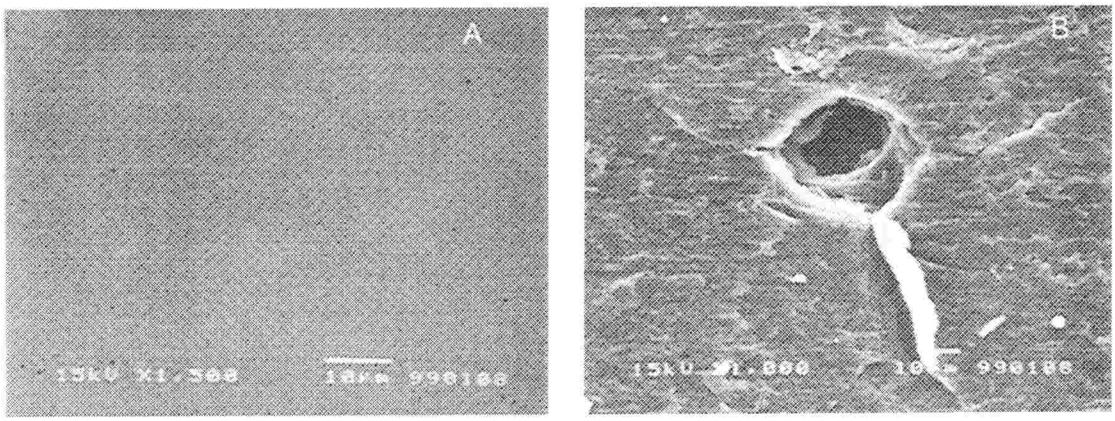

Fig. 3.7. SEM observation of the surface of PCL plastic films.
A: before the degradation, B: after the 25-day burial in the BVL.

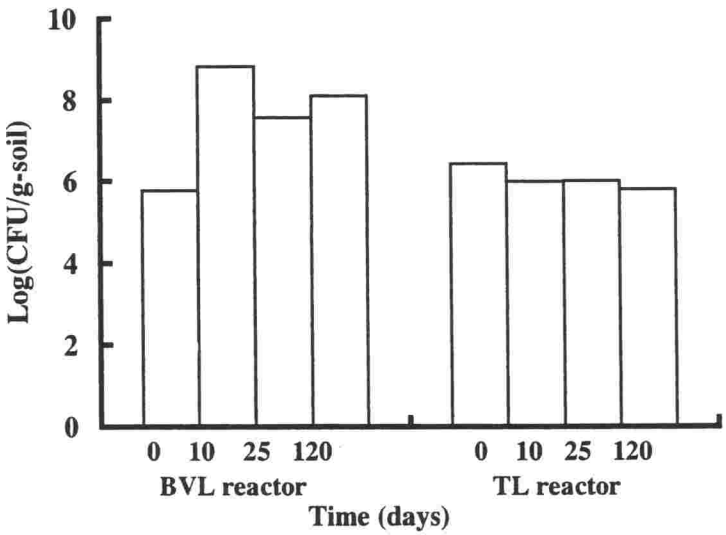

Fig. 3.8 Population of PCL degrader in TL and BVL reactors.

3.3.3. Degradation of starch-PVA blend plastic

Fig. 3.9 shows the changes in the weight loss and film thickness of starch-PVA plastic in the TL and BVL reactors. The weight loss of the starch-PVA plastic in the TL gradually increased and reached 52 % after 120days. On the other hand, in the BVL, the rate of weight loss for the initial 30days was slightly higher than that in the TL. However the final weight loss in the TL and BVL reactors were almost the same. Although the bioventing enhanced the initial degradation of starch-PVA, final degradability was not affected by the bioventing. Film

thickness of the starch-PVA in the BVL immediately decreased from 32μm to 21μm in the initial 10days, but the final thickness in the BVL was not different from that in the TL. Although 50% weight loss occurred, breakage on the film had not been observed in the both reactors (Fig. 3.10). From the SEM observation (Fig. 3.11), it was observed that the smoothness on the film surface disappeared within 120-day burial in both reactors, indicating the removal of starch. Fig. 3.12 shows the population sizes of PVA- and starch-degraders in the reactors. Population of the PVA-degrader was considerably lower than that of starch-degrader. Population of starch-degrader in the BVL reactor was a little higher than that in the TL reactor, indicating the bacterial stimulation by the bioventing.

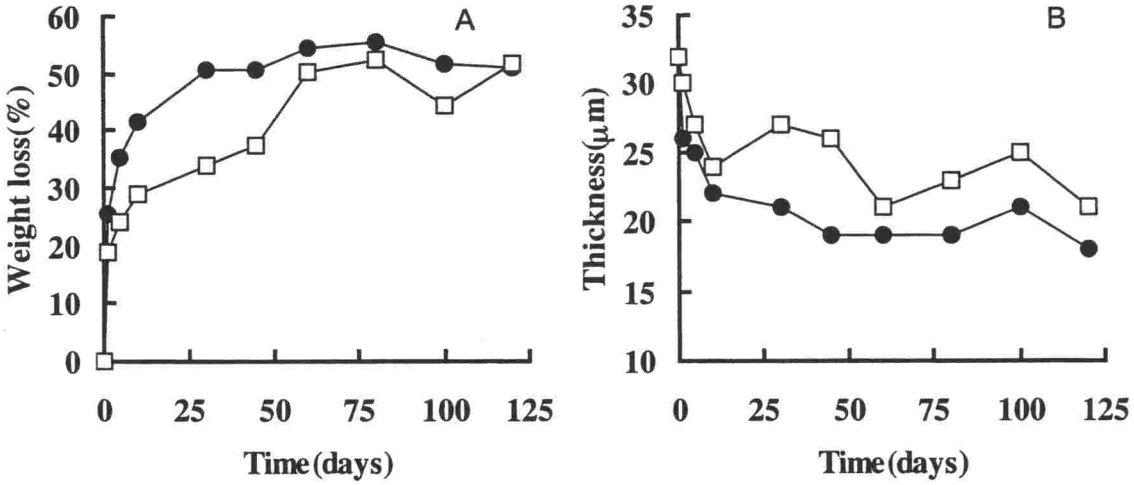

Fig. 3.9. Degradation of starch-PVA plastic film in the TL (□) and BVL (●) reactors.
A: weight loss of film, B: film thickness

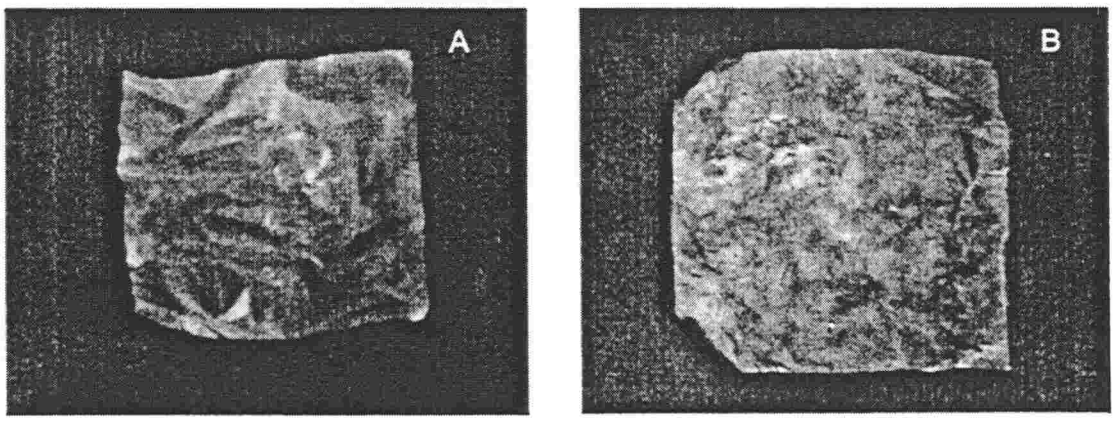

Fig. 3.10. Starch-PVA plastic film degraded in the reactors
A: after 120-day degradation in TL, B: after 120-day degradation in BVL

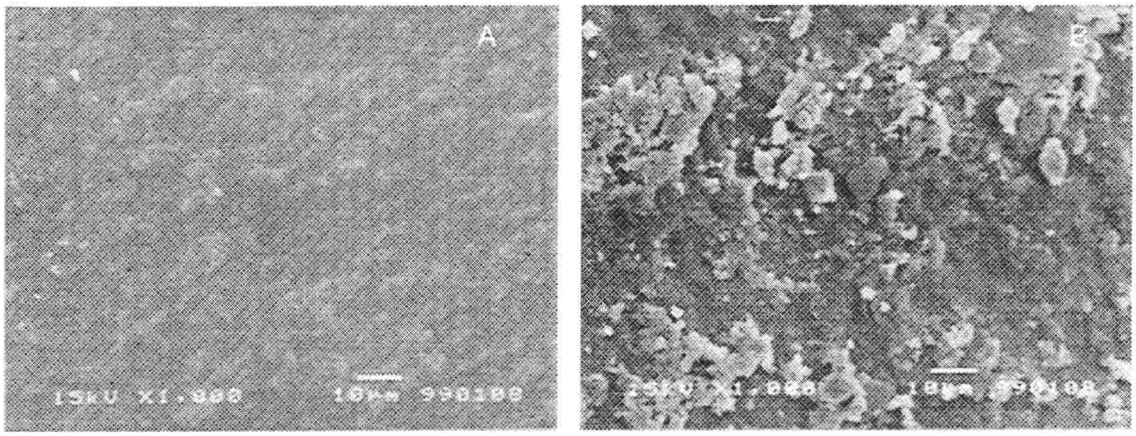

Fig. 3.11. SEM observation of the surface of starch-PVA plastic films.
A: before the degradation, B: after the 120-day burial in BVL.

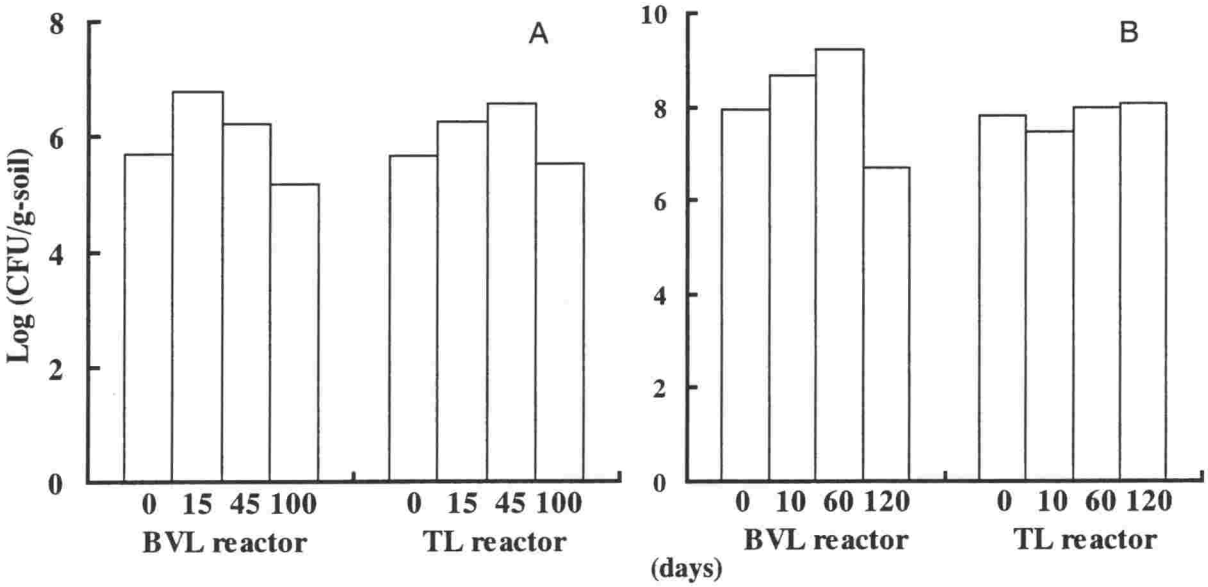

Fig. 3.12 Population of PVA- and starch-degrader in the TL and BVL reactors. A: population of PVA-degraders, B: population of starch-degraders

3.4. Degradation of CA plastic

Fig. 3.13 shows the changes in the weight loss and film thickness of the CA plastic in the TL and BVL reactors. Degradation behavior of the CA plastic was similar with that of starch-PVA blend film. Higher initial degradation rate in the BVL was obtained, though the final degradability were not so different in both reactors, approximately 30%. Any breakages on the films had been never seen in both reactors (Fig. 3.14). Film got wrinkled and it turned to light

brown or yellow after 80 days. SEM observation revealed that the film surface was partially eroded (Fig. 3.15) in the BVL, and similar observation was done in the TL reactor. Fig. 3.16 shows the population changes of the CA degrading microbes in the TL and BVL reactors. In both reactors, population of CA-degrader decreased from 40days and the population sizes were not so different between TL and BVL reactors.

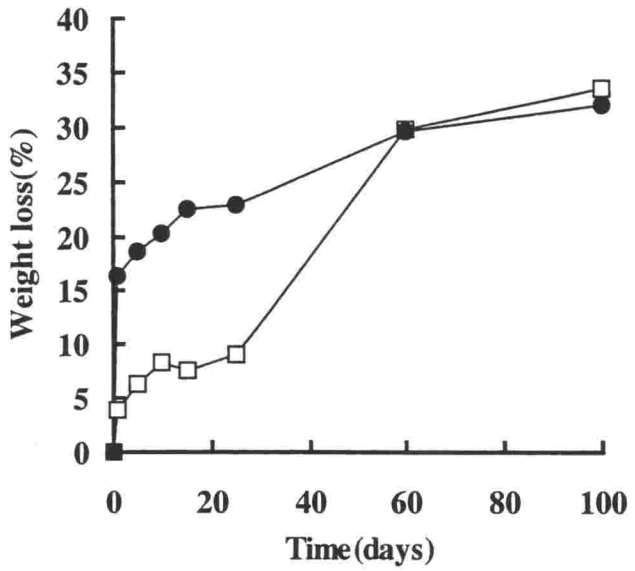

Fig.3.13 Weight loss of CA plastic film in TL (□) and BVL (●) reactors.

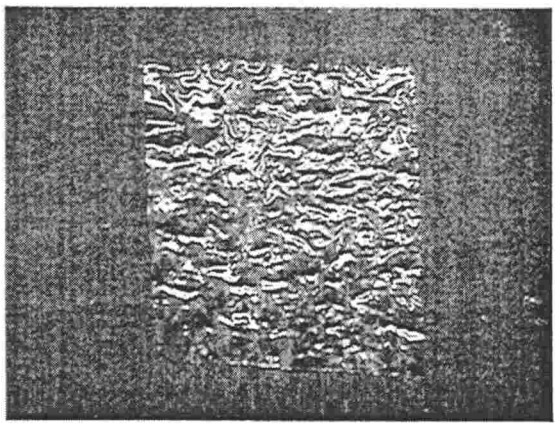

Fig.3.14 CA plastic film after the 120-day degradation in BVL reactor.

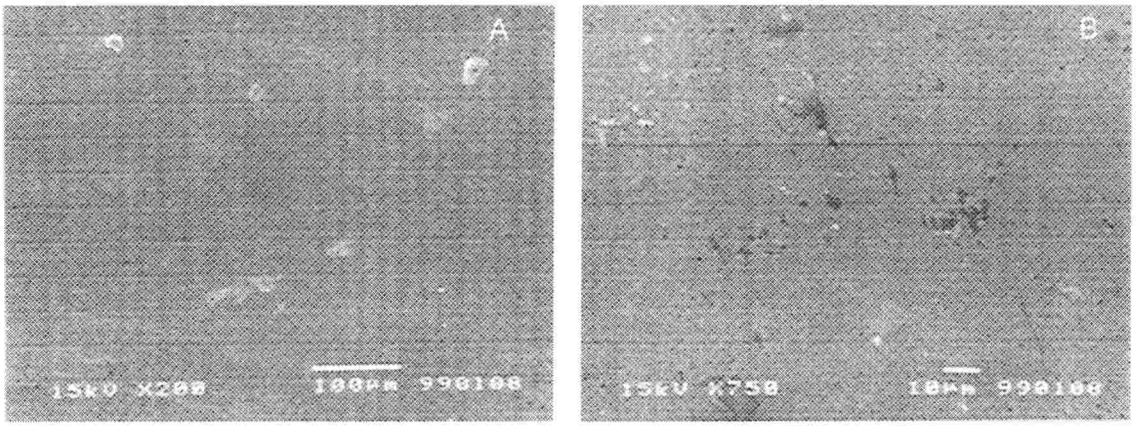

Fig. 3.15. SEM observation of the surface of CA plastic films.
A: before the degradation, B: after the 120-day burial in BVL.

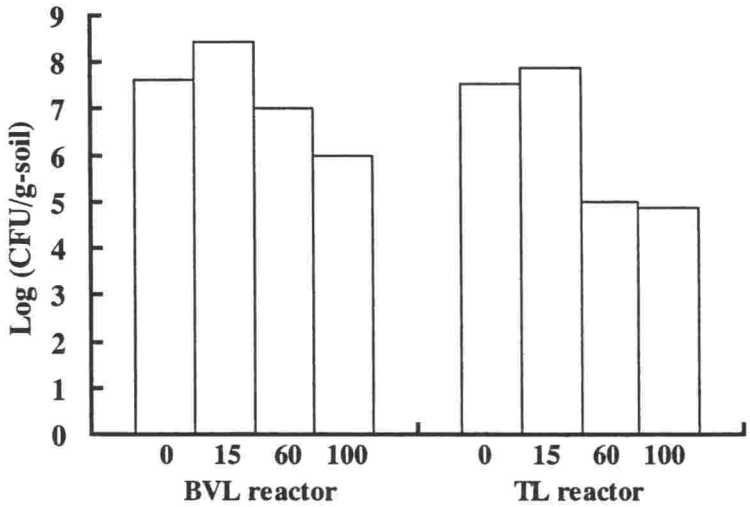

Fig. 3.16. Population of CA degrader in TL and BVL reactors.

3.4. DISCUSSION

In this study, we evaluated the degradation of the plastics by measuring its weight loss and observing the film form by SEM. Since the weight loss refers to the erosion of molecules from the solid phase into the aqueous phase, the value does not always indicate complete degradation or mineralization of the molecules. However, the molecules released from plastic

film might be theoretically easily degraded low molecular weight compound such as organic acids and ketonic compounds (Suzuki *et al.* 1978, Sakai *et al.* 1985). These dissolved components are not recalcitrant, and would be easily degraded by microorganisms in natural environment. Therefore, the evaluation of its degradation based on weight loss seems practical.

BVL has been expecting as a technique for promoting a stabilization or settlement of waste landfill. In this chapter, possibility of the enhanced *in-situ* degradation of plastics by applying BVL was investigated using small-scale model landfill reactors. An apparently enhanced degradation of PHB/V film was observed in the BVL reactor compared with the TL reactor. In BVL reactor, PHB/V completely lost the weight after 100 days and the film was broken into small species, while no significant weight loss occurred in TL reactor. The bioventing could stimulate the growth and activity of PHB-degrader, and led to the enhanced PHB/V degradation. On the other hand, although population of PCL-degrader also increased by applying the bioventing, degradation of PCL was not enhanced in the BVL reactor, and the weight loss proceeded a little more rapidly in the TL reactor than in the BVL reactor. One possible reason for the poor PCL degradation in the BVL reactor is probably its environmental condition, e.g. pH. According to the previous report (Sawada 1993), an acidic environment caused the acceleration of PCL hydrolysis. Since the TL was under the anaerobic condition, (pH value of the waste suspension was below 5 or 6; data not shown), a rapidly degradation of PCL might be proceeded. Finally a remarkable film breakage started after 40 days and film had lost the surface luminescence in both reactors. From the viewpoint of landfill settlement, PHB/V and PCL plastics would be applicable for the volume reduction of plastic waste, because it could be fragmented and will be completely degraded even without bioventing.

Initial degradation rates of starch-PVA blend and CA within the BVL reactor were faster than those within the TL reactor while final degradability was not so different between both

reactors and film breakage of these films had never been observed. In the case of degradation of starch-PVA blend, a previous study reported that degradation of the PVA component was necessary for the film breakage (Ishigaki *et al.* 1999). Since starch content of the film examined in this study was approximately 60 %, considerable portion of starch would be degraded and it was enhanced by the bioventing. However, it could not stimulate the PVA degradation in this experiment. In the case of CA degradation, it has been reported that CA solubility and degradability depend on their degree of acetyl substitution (Reese 1957). In our preliminary study, CA film was dissolved to sterilized water and the film lost 25% of its weight in 28 days. Solubilized fraction was suggested to be lower-substituted CA and highly-substituted CA would be still remaining in the film. Therefore degradation of the highly substituted fraction would be needed for further degradation and decomposition of CA film. Judging from the experimental results, the bioventing seemed to accelerate the solubilization and/or biodegradation of the lower-substituted fraction of CA, but did not lead to the enhancement of degradation of highly-substituted fraction.

From the experimental results obtained in this chapter, it may be concluded that the application of bioventing to the landfill can considerably stimulates the growth and activity of certain groups of PDMs (e.g. PDMs for PHB, starch and lower-substituted CA) and, consequently, can enhance the degradation of certain biodegradable plastics. For example, when the bioventing is applied to the landfill coupled with the use of the PHB/V plastic, more rapid or efficient settlement of the landfill might be achieved. On the other hand, bioventing may not contribute much to accelerate the volume reduction of starch-PVA blend and CA plastics, because the fragmentation or complete degradation of these plastic films did not occur even in the BVL reactor. To achieve the fragmentation of these plastics, stimulation of the microbial population which can degrade recalcitrant parts of the plastics, i.e. PVA and highly-substituted

CA, would be requisite. Since such microbial populations are considered to exist rarely in the landfill site, their bioaugmentation seems necessary to achieve the enhanced in-situ degradation of these plastics.

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Chapter 4
Biodegradation of a Starch - Polyvinyl Alcohol Blend Plastic Film

4.1. INTRODUCTION

It was indicated in Chapters 2 and 3 that PDMs widely existed in waste landfill sites and landfill has a certain potential for plastic degradation. However, in Chapter 3, stimulation using bioventing could not enhanced the degradation of some plastics e.g. starch-PVA blend or CA, despite of the presence of corresponding PDMs in the reactors. Regarding these plastics, clarification of the features of degrading bacteria or detailed degradation mechanism is necessary to find out the inhibiting factor of the degradation and to develop the strategy for the enhanced biodegradation.

This chapter deals with the biodegradation of starch-PVA blend plastic which was not degraded in Chapter 3. Low biodegradability of starch-PVA blend plastic compared with other biodegradable plastics has been previously reported also by other researchers. Bastioli *et al.* (1992, 1993) reported that an amylose-PVA composite (starch-PVA blend) was biodegraded very slowly and that 75 % weight loss required 300 days in a degradation test with activated sludge. Iwanami *et al.* (1992) reported that the weight loss of starch-PVA blend at 50 % in a waste composting process. In this chapter, the degradation mechanism of a commercial starch-PVA blend plastic was examined. Based on the results, feasibility of bioaugmentation or

enzymatic pretreatment has been discussed as a strategy for enhanced degradation of the plastic.

4.2. MATERIALS AND METHODS

4.2.1. Plastic film

Mater-bi AF10H (industrial grade for film blowing) (Iwanami *et al.* 1992) was used as a Starch-PVA blend plastic. Mater-bi AF10H contains approximately 60% starch and natural additives (starch fraction), and approximately 40% of modified PVA and plasticizers (PVA fraction). It was used in the form of square film (50 mm x 50 mm), with a thickness of 30μm.

4.2.2. Activated sludge and bacterial strains

An activated sludge which had been acclimated to synthetic wastewater (Furukawa *et al.* 1994) by the fill and draw operation was used for the biodegradation tests. *Bacillus subtilis* strain X isolated from soil by our laboratory was used as a starch-degrading bacterium (isolation and identification data not shown). Strain X has no PVA-degrading activity. *Pseudomonas vesicularis* var. *povalolytics* strain PH (Fujita *et al.* 1985) isolated from a PVA-acclimated activated sludge was used as a PVA-degrading bacterium. The optimal condition for growth of strain PH is pH 7.5 at 30 °C (Kawagoshi *et al.* 1996), and it has no starch degrading activity.

4.2.3. Enzymes

Commercially available α-amylase (Wako Pure Chemicals Industries Ltd., Japan) was used as a starch-degrading enzyme. The PVA-degrading enzyme was extracted from the culture broth of strain PH, partially purified by salt-precipitation with ammonium sulfate and dialysis (Kawagoshi *et al.* 1996), and used as a crude sample. The optimal condition of the enzyme activity is pH 9.0 at 40 °C. This crude enzyme sample showed both activities of PVA-oxidase and PVA-hydrolase (Kawagoshi *et al.* 1997).

4.2.4. Degradation test by activated sludge

A 300-ml Erlenmeyer flask containing the plastic film and 100 ml of activated sludge, of which mixed liquor suspended solid (MLSS) concentration was adjusted to 50, 500 or 2000 mg/l by dilution with deionized water, was incubated at 28 °C and 120 rpm on a rotary shaker. As a control, 100ml of sterilized deionized water was used instead of the activated sludge. A definite volume (0.2, 2 or 8 ml) of synthetic wastewater mainly composed of meat extract and peptone (Furukawa *et al.* 1994) was added to the flask at the start of the degradation test. In some experiments, an appropriate amount of the synthetic wastewater was periodically added during the degradation test. During the incubation, the weight of the film was periodically measured as described below for evaluating its degradation. The degradation tests were carried out in duplicate for each condition. All standard errors were less than 2.0 % and the results showed good reproducibility.

4.2.5. Degradation test by bacterial strains

The starch-degrading bacterium strain X was cultivated in the starch medium (K_2HPO_4: 1 g/l, $(NH_4)_2SO_4$: 1 g/l, $MgSO_47H_2O$: 200 mg/l, $FeCl_3$: 10 mg/l, NaCl: 50 mg/l, $CaCl_2$: 50 mg/l, soluble starch (derived from potato, Wako) 1000 mg/l), and the PVA-degrading bacterium strain PH was cultivated in PVA-TY medium (Fujita *et al.*1985) at 28 °C and 120 rpm on a rotary shaker. The plastic film and 5 ml of each bacterial culture at mid-log phase were added to 100 ml of fresh medium (starch medium or PVA-TY medium) in a 300-ml Erlenmeyer flask, and incubated at 28 °C and 120 rpm on a rotary shaker. During the incubation, the weight of the film was periodically measured in duplicate experiments. All standard errors were less than 1.5 % and the results showed good reproducibility.

4.2.6. Degradation test by enzymes

To one 300-ml Erlenmeyer flask were added 300 units of α-amylase, which were adjusted to initial starch-degrading activity of strain X, suspended in 100 ml of phosphate buffer (pH7.5) and the plastic film. The flasks were incubated at 30 °C and 120 rpm on a rotary shaker. On the other hand, to another 300-ml Erlenmeyer flask were added PVA-degrading crude enzyme, which was prepared from 100ml of the culture broth of strain PH in PVA-TY medium, suspended in 100 ml of Tris buffer (pH 7.5 or 9.0) and the plastic film. The degradation tests were carried out at 120 rpm on a rotary shaker, at pH 7.5 or pH 9.0 at 30°C or 40°C. This optimal conditions for growth of strain PH was pH 7.5 at 30 °C, and are that for the enzyme activity was pH 9.0 and 40 °C. During the incubation, the weight loss of the plastic film was periodically measured in duplicate experiments. All standard errors were less than 2.5 % and the results showed good reproducibility.

4.2.7. Determination of weight loss of Mater-bi film

The plastic film was taken from the test flask, washed with deionized water, dried for 30 min in a desiccator, and its wet weight was measured. After the measurement, the film was returned into the test flask, and the degradation test was continued. When the film was broken into small pieces, they were collected on a 24-mesh sieve for weight measurement. In preliminary tests, the proportionality between the wet weight (measured by this method) and dry weight of the plastic film in the degradation process was confirmed (proportional value was 0.68 and correlation coefficient was 0.991).

4.2.8. Enzyme assays

Enzyme activities in the medium or buffer of degradation tests were periodically assayed. The

α-amylase was assayed by the method of Noelting (1948). The sample and starch solution were put into an L-type test tube to give a total volume of 10 ml and the final starch concentration of 1 g/l. This reaction mixture was incubated for 20 min at 40 °C and 75 rpm on a reciprocal shaker. The sugar produced was measured by the absorbance at 520 nm according to the Somgyi-Nelson method (Nelson 1944), and the α-amylase activity was calculated in terms of micromoles of reduced sugar produced in 1 minute as a Unit. The activity of the PVA-degrading enzyme was assayed as previously described (Kawagoshi *et al.* 1996). The sample and PVA solution were put into an L-type test tube to give a total volume of 20 ml and a final PVA concentration of 2 g/l. This reaction mixture was incubated for 2 hours at 28 °C and 70 rpm on a reciprocal shaker. The residual PVA concentration after enzymatic degradation was measured by the absorbance at 690 nm according to the Finley's iodine colorimetric method (Finley 1961), and the enzyme activity was expressed in terms of micromoles of PVA decreased in 1 minute as a Unit.

4.2.9. Scanning electron microscopy (SEM) observation

Film samples were coated with gold and the surface was investigated with a JEOL 5410LV scanning electron microscope.

4.3. RESULTS

4.3.1. Degradation by activated sludge

In order to optimize the biodegradation of starch-PVA blend plastic by activated sludge, we examined the effects of initial microbial density (MLSS concentration, 50 to 2000 mg/l) and of microbial activation by sequential addition of synthetic wastewater. Typical results are shown in Fig. 4.1. The film suspended in sterile deionized water as a control showed a slight weight loss. Autoclaved activated sludge gave similar results (data not shown). Starch was detected in

the sterilized deionized water after the control indicating that a part of the starch fraction of this plastic had been dissolved into the aqueous phase. The most efficient weight loss of the film was observed at the initial MLSS concentration of 500 mg/l with the addition of 2ml of synthetic wastewater in every other day. However, even under this optimized condition, the ultimate weight loss was approximately 40 % or less. Prolonged incubation to about 3 months did not lead to further weight loss. Furthermore, no breakage of the film was observed during the degradation tests, although the film became thinner than the original thickness based on visual observation (Fig. 4.2A and 4.2B). Degradation in activated sludge obtained from another practical municipal sewage treatment plant showed the similar results: the ultimate weight loss was 45 % and no breakage of the film was observed.

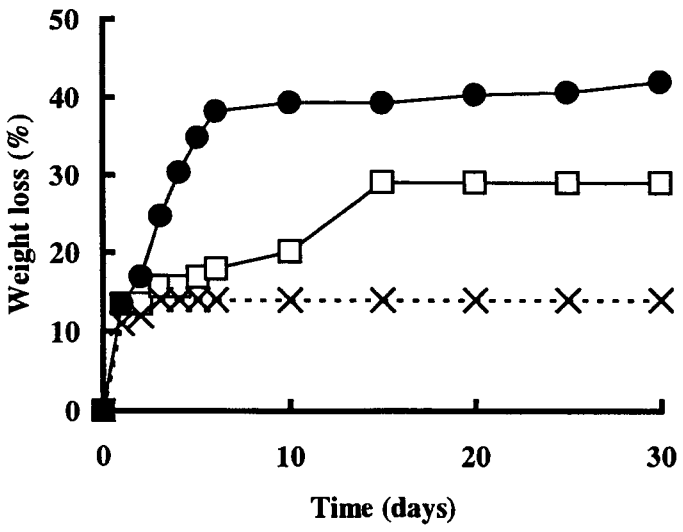

Fig. 4.1. Weight loss of PVA-starch plastic film by activated sludge (initial MLSS=500mg/l). ●: with 2ml of synthetic wastewater added, □: without synthetic wastewater and ×: control (sterile deionized water).

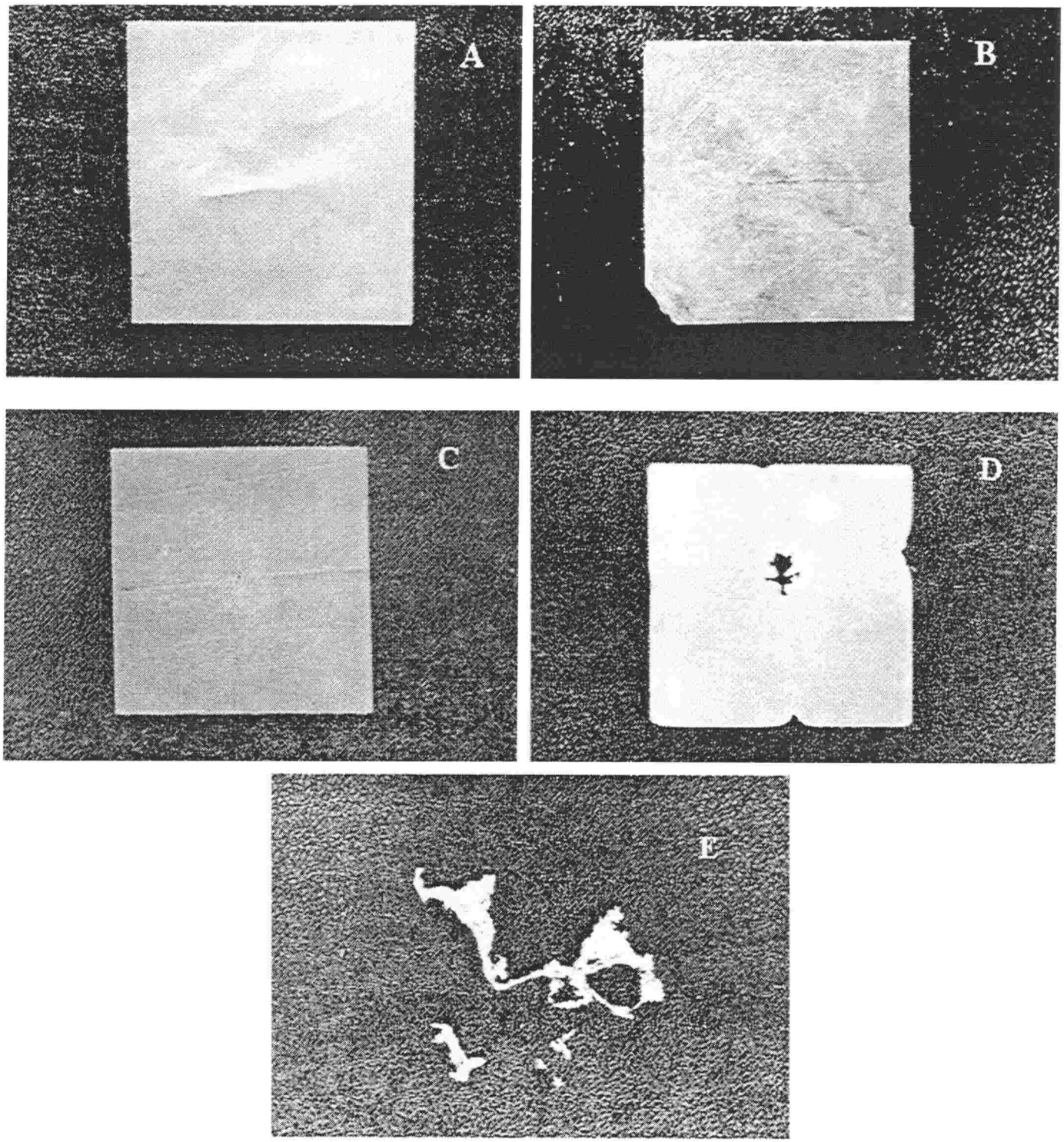

Fig. 4.2. Pictures of starch-PVA film samples before the degradation test (**A**), and after the degradation test by activated sludge (**B**), by *B.subtilis* strain X (**C**), by *P.vesiclaris* strain PH (**D**), and by PVA-degrading enzyme (**E**)

4.3.2. Degradation by *B.subtilis* strain X

Fig. 4.3A shows the results of the degradation by the starch-degrading bacterium strain X. The weight loss proceeded more rapidly than that in using activated sludge, and the ultimate weight loss was 45 % within 5 days. However, breakage of the film was not observed as in the case of degradation by activated sludge (Fig. 4.2C).

4.3.3. Degradation by α–amylase

The weight loss in the degradation test using α-amylase proceeded more rapidly than in that using strain X and the ultimate weight loss reached approximately 50 % at day 1 (Fig. 4.3B). However, no breakage of the film occurred (photo not shown). Although α-amylase activity was maintained at relatively high levels throughout the degradation test, no further weight loss occurred after prolonged incubation.

4.3.4. Degradation test by *P.vesicularis* strain PH

Fig. 4.4 shows the results of the degradation using the PVA-degrading bacterium strain PH. PVA-degrading activity of the culture increased with the cell growth, and reached a maximum at day 10, followed by a gentle decrease. The weight loss of the film reached 48.5 % at day 30. Despite the lower enzymatic activity after day 10, a gradual weight loss continued upto day 30, resulting in the ultimate weight loss of 50 %. Different from the previous experiments, film breakage from the edge and center was observed after day 15 (Fig. 4.2D).

4.3.5. Degradation by a PVA-degrading enzyme

Fig. 4.5 shows a representative time course of the weight loss of the plastic film and PVA

degradation activity in the test buffer. Table 4.1 summarizes the weight loss at day 10. The weight loss was greatest at pH 9.0 at 30 °C, reaching approximately 70 % at day 10. Film breakage also began at day 4, and the film broke into small pieces within 10 days (Fig. 4.2E). As shown in Fig. 4.6, the PVA-degrading activity of the test buffer tended to decrease with further weight loss.

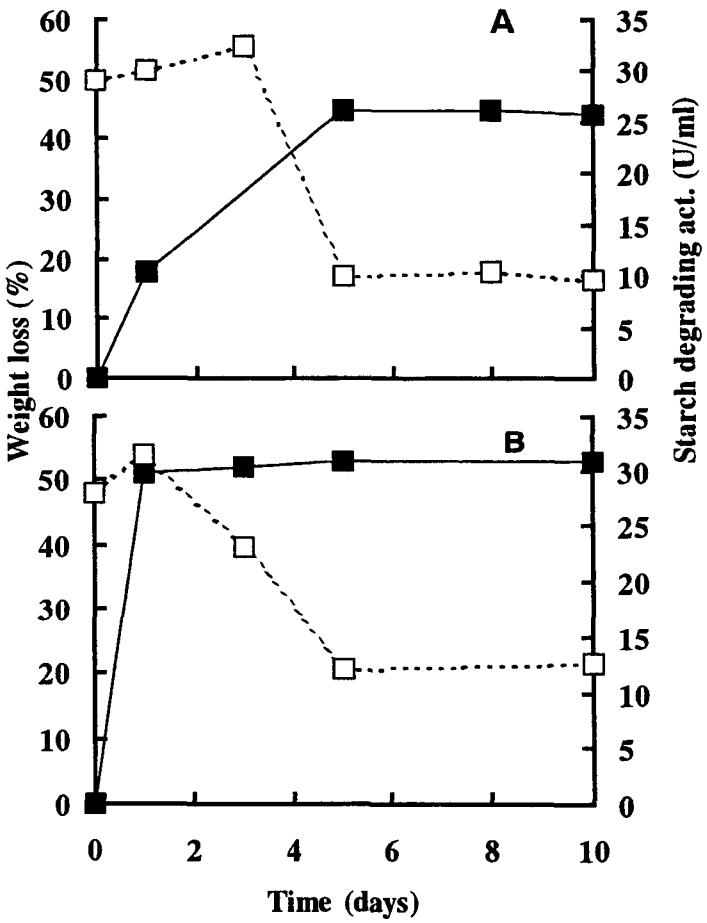

Fig. 4.3. Weight loss of PVA-starch plastic film by (A) starch degrading bacterium *B.subtilis* strain X and (B) α-amylase. ■:weight loss, □:enzyme activity

4.3.6. SEM observation of the degraded film surfaces

Fig. 4.6A-D shows SEM views of the surface of the plastic film after the degradation test. Although the film before the tests had a smooth surface and many starch particles were observed (Fig. 4.6A), a rough surface and partial disappearance of starch particles were observed after degradation by activated sludge (Fig. 4.6B). Further degradation of starch particles was observed after the treatment with the starch-degrading bacterium (strain X) or enzyme (α-amylase). After the degradation test, the film surface appeared uneven due to the complexity of the PVA skeleton was revealed clearly (Fig. 4.6C). On the other hand, in the samples degraded by the PVA-degrading bacterium (strain PH) or enzyme, partial destruction of the PVA skeleton was observed (Fig. 4.6D), and the inner starch granules were exposed.

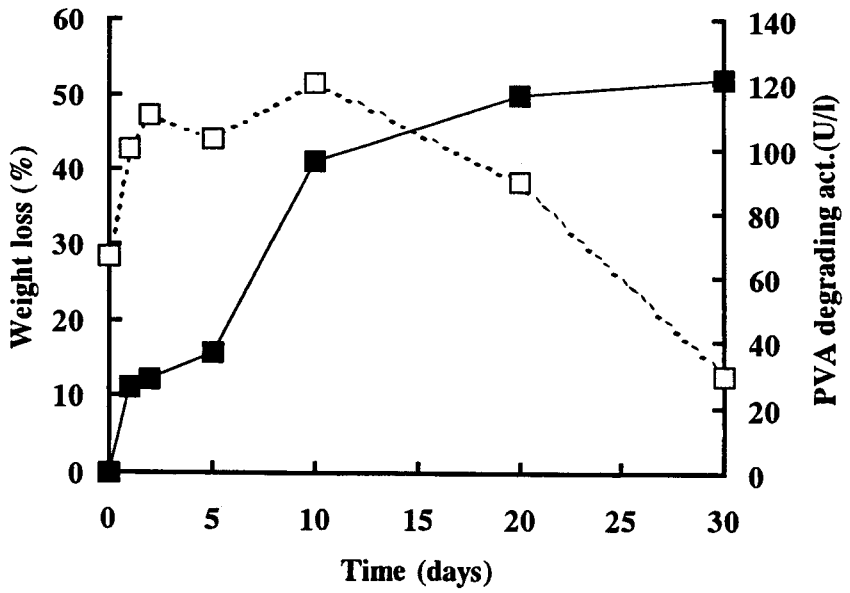

Fig. 4.4. Weight loss of PVA-starch plastic film caused by PVA degrading bacterium *P.vesiclaris* strain PH. ■:weight loss, □:enzyme activity

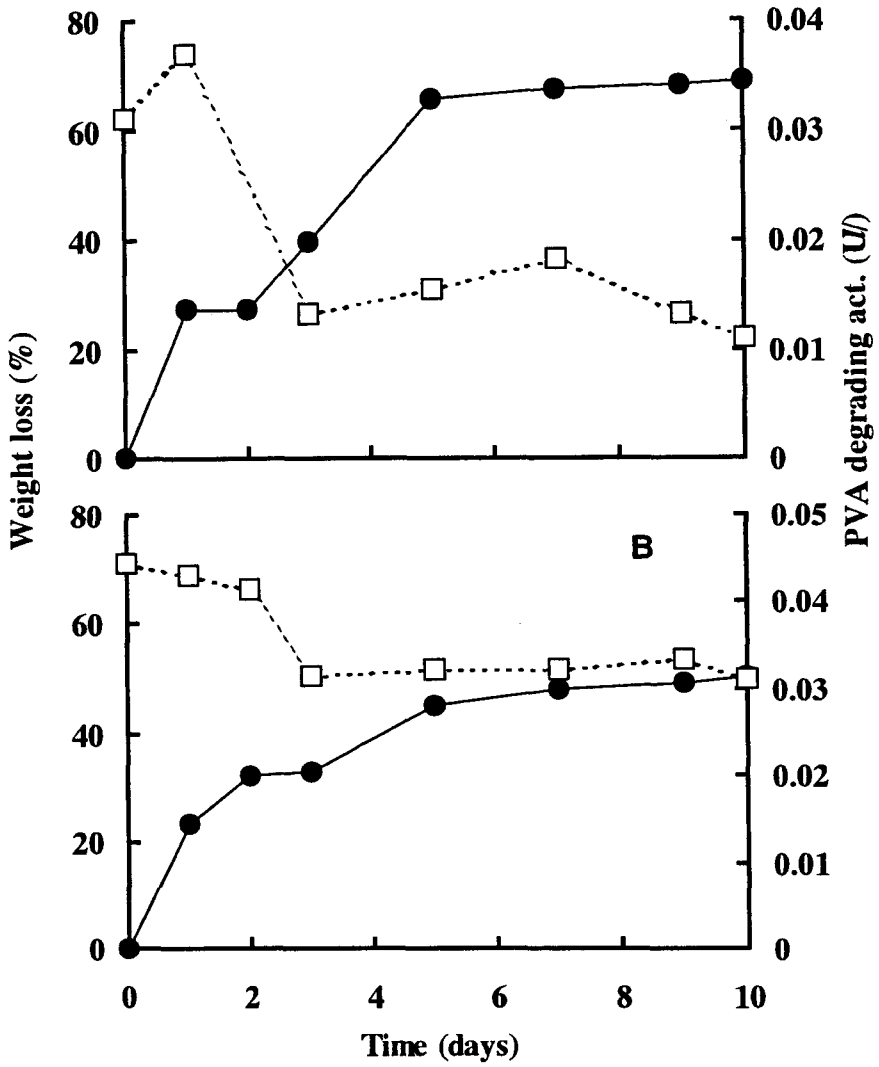

Fig. 4.5. Weight loss of PVA-starch plastic film caused by PVA-degrading enzyme under (A) 30℃, pH9.0 and (B) 30℃, pH7.5. ● weight loss, □: enzyme activity

Table 4.1. Degradation of the PVA-starch plastic by PVA-degrading enzyme after a 10-day incubation

Temp.	pH	Weight loss (%)	Fragmentation*
30°C	7.5	50.0	+
30°C	9.0	69.0	++
40°C	7.5	51.5	+
40°C	9.0	45.5	-

*Fragmentation of the film was evaluated by the following criteria.
-:no film breakage was observed
+:film breakage was observed
++:remarkable film breakage was observed

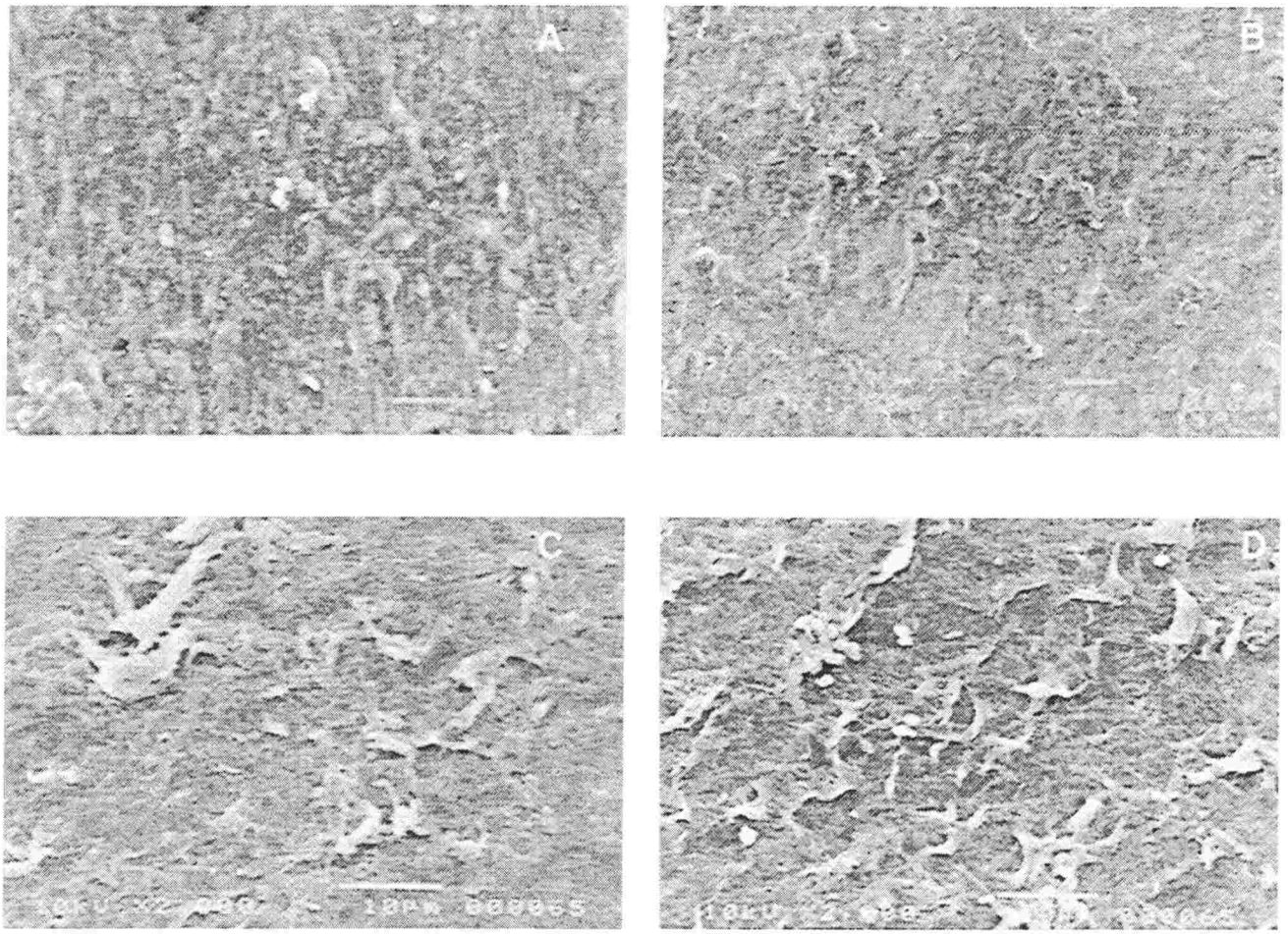

Fig. 4.6. SEM pictures of the surface of starch-PVA film samples. Before the degradation test (**A**) and after the degradation test by activated sludge (**B**) by α-amylase (**C**) by PVA-degrading enzyme (**D**).

4.4. DISCUSSION

In the control degradation test in the sterilized deionized water, a part of the starch fraction of this plastic was dissolved into the aqueous phase. The degradation test using activated sludge was carried out for simulating the degradation in a natural environment such as a landfill sites. Although the addition of appropriate nutrients to activate microbes was effective for accelerating initial weight loss, the ultimate weight loss did not exceed 40% and breakage of the film did not occur even after three months under an optimized condition. Therefore, this starch-PVA blend plastic film may not be easily degraded in natural ambient conditions. If we make no effort to promote the degradation, fragmentation into small pieces or molecules will not proceed and the film form will remain for an extremely long period.

Thus, we examined the degradation mechanism by treatment with bacterial cultures and/or enzymes which can catalyze the biodegradation of starch or PVA, which are the two main components of this plastic. In the presence of a starch-degrading bacterium and enzyme (α-amylase), the rate of the initial weight loss was increased compared with that using activated sludge. However, the ultimate weight loss was about 50 % (less than the starch content of this plastic, 60%), and breakage of the film did not occur. On the other hand, application of the PVA-degrading bacterium and/or enzyme resulted in a maximal weight loss of about 70% and film breakage. Application of the enzyme was especially effective, and led to the film fragmentation into small pieces within 10 days under pH 9.0 at 30 °C. Since this plastic contains 40% PVA, it is apparent that not only the PVA fraction but also a considerable portion of the starch fraction was lost from the film by the treatment with PVA-degrading enzyme. As the PVA-degrading bacterium and enzyme used here showed no starch-degrading activity, loss of the starch fraction seems to be the result of its dissolution with the degradation of the PVA fraction. These experimental results indicated that degradation of the PVA fraction is important

and requisite for complete degradation or decomposition of this plastic film.

Tokiwa *et al.* (1992) reported that they could obtain PVA-degrading bacteria from only 4 soil samples screened out of 100, and concluded that PVA-degrading bacteria rarely exist in a natural environment. On the other hand, Hashimoto *et al.* (1980) reported that bacteria capable of degrading PVA were commonly distributed in various environments but that a long period was necessary to enrich them to exhibit apparent PVA-degrading activity. Limited and slow degradation in a natural environment and during the composting processes may depend on such difficulties in utilizing naturally-distributing PVA-degrading bacteria or enzymes. Thus the application of PVA-degrading bacteria or enzymes prepared in laboratories, seems very useful for degradation.

More rapid weight loss and destruction of film form were observed when enzyme solutions were applied than when bacterial cultures were used for the degradation tests. The weight loss caused by α-amylase was approximately 50 % after a 1-day incubation, while that by the starch-degrading bacterium strain X was 48 % after a 5-day cultivation. The weight loss caused by the PVA-degrading crude enzyme was 65 % after 5 days, although the weight loss by the PVA-degrading bacterium strain PH was 52 % after 1 month. The enzyme application gave superior results probably because: (i) degrading activity was high from the beginning of the degradation tests with enzyme solutions, whereas there was a lag time in bacterial enzyme production, (ii) the conditions were optimal for enzymatic degradation activity but not for bacterial growth, (iii) the bacteria take time to adhere to the plastic films before biodegradation (Imam and Gould 1990), whereas the enzyme acts immediately upon contact and (iv) enzymes would easily permeate into the film, although bacterial cells could not.

Based on these results, degradation of PVA is requisite for enhanced fragmentation of starch-PVA plastic and enzyme application would be possible to improve the in situ degradation

of this plastic. Bioaugmentation has been often researched but rarely implemented successfully. There are numerous reasons for difficulties in the use of introduced bacteria, i.e. growth inhibition of introduced bacteria caused by competition with indigenous bacteria (Stephenson and Stephenson 1992). Thus enzyme application seems better to improve the *in-situ* degradability of plastic. Furthermore, since starch-PVA plastic would be widely applied in agricultural or fishery field, they can be easily separated at the elimination source and solely collected. It is also suggested the evaluation of enzymatic pretreatment process before landfilling is important.

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Chapter 5

Enzymatic Degradation of Cellulose Acetate Plastic by Novel Degrading Bacterium *Bacillus* sp. S2055

5.1. INTRODUCTION

As shown in Chapter 3, degradation/fragmentation of CA was not proceeded by stimulation of indigenous bacteria as well as starch-PVA blend. Such extremely slow degradation of CA has been also reported by previous researchers. For example, the degradation of CA plastic film (degree of substitution (DS) 2.0) took several years in many water and soil samples (Ach 1993). On the other hand, there has been several reports demonstrating relatively rapid CA degradation as well. Gross and Gu (1995) reported that a powdery CA (DS 1.7) showed 72.4 % of mineralization within 24 days in a composting reactor. Buchanan *et al.* (1993) also reported that a CA film (DS 2.5) was degraded by activated sludge up to 40% of the initial weight during 70 days. These contradictory results suggested that efficiency of CA plastic degradation strongly depends on the numbers and characteristics of CA-degrading microbes and the environmental conditions.

Despite many reports concerning the CA degradation under natural or semi-natural environments, only two articles have described about bacterial strains capable degrading CA plastics in pure cultures within our reference search. Nelson *et al.* (1993) isolated a *Pseudomonas paucimobilis* which can utilize CA films (DS 1.7 and 2.5) as a sole carbon source

for its growth, but the degradation mechanism was not well described. Sakai *et al.* (1998) isolated two bacterial strains of *Neisseria sicca*, SB and SC, capable of degrading a CA textile (DS 2.34) and CA membrane filters (DS mixture of 2.8 and 2.0). The CA membrane filter was fragmented into pieces during 10-day cultivation. It was also demonstrated that CA degradation by these strains led to the release of acetate and reducing sugar and, therefore, the production of esterase(s) and cellulase(s) related to the CA degradation was suggested. Still little is known about the microbes and enzymes responsible for CA biodegradation up to date. Especially knowledge concerning the property of CA-degrading enzyme(s) is very scarce. Although Reese (1957) has reported that a fungal strain, *Pestalotiopsis westerdikii* QM381, produced an esterase which deacetylates cellobiose octaacete (CBOA) and water-soluble CA (DS 0.76), it was inactive against water-insoluble CA plastics. Therefore, further detailed studies on the degrading microbes and enzymes are necessary as to clarify the potentials and mechanisms of CA biodegradation and, consequently, to extend the application fields of this plastic.

In this chapter firstly describes the natural abundance of CA-degrading bacteria and their taxonomical characteristics. Subsequently, partial characterization of CA-degrading enzyme(s) in *Bacillus* sp. strain S2055, which was isolated from a waste disposal landfill leachate as a most efficient CA-degrader, was tried.

5.2. MATERIALS AND METHODS

5.2.1. Plastic

A CA plastic film with average DS 1.7 (industrial grade for film blowing) was obtained from Daicel Chemical Ind. Ltd. It was used for the degradation tests in the form of square film (50 mm x 50 mm). Powdery CA with DS 3.0 and DS 1.5 were purchased from Acros Organics Co. Ltd. (Geel Belgium) and Kishida Chemical Co. Ltd. (Osaka, Japan), respectively, and used

for preparing CA-containing media.

5.2.2. Enumeration, isolation and taxonomical characterization of CA-degrading bacteria

Enumeration of CA-degrading bacteria was performed using the emulsified CA medium by the plate count technique. Samples were plated onto the medium after appropriate dilution (soil samples were firstly suspended in sterile deionized water) and the plates were cultivated at 28 °C for 10 days. Resultant colonies with halos, which indicate the CA degradation in the medium, were counted as CA-degrading bacteria here. Colonies showing clear halos were picked up and isolated as the CA-degrading bacteria. The isolated CA-degrading bacteria were morphologically and physiologically characterized and identified up to genus level according to Cowan and Steel (1973) and Bergey's Manual (1984). Bacterial identification kits (API20NE and/or API20E; Bio Merieux S.A., L'etoile, France) were also used for ensuring the identification of gram-negative bacteria.

5.2.3. Enzymes

The crude preparation of CA-degrading enzyme(s) was made from the cell-free supernatant of the 5-day culture of *Bacillus* sp. strain S2055 by salt-precipitation with ammonium sulfate and dialysis (Kawagoshi *et al.* 1996). When required, 3 major protein fractions of this crude enzyme were fractionated using a high performance liquid chromatography with a gel permeation column (GPC: TSK-GEL G4000SW, 7.5 mm x 300 mm, Tosoh Co., Tokyo) with an eluent of 50 mM sodium phosphate buffer (pH 7.0) at flow rate of 1.0 ml/min. Commercially-available cellulase (*Aspergillus niger*), lipases (*Rhizopus arrhizus*, *Pseudomonas* sp.), and esterase (porcine liver) were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan), while another lipase (*Candida rugosa*) from Wako Pure Chemical Ind. Ltd.

(Osaka, Japan). These enzymes were used as references for characterizing the CA-degrading enzyme(s) extracted from strain S2055.

5.2.4. CA plastic degradation test by bacterial strains

CA-degrading microbes isolated from various environment samples were cultivated in the CA-GPY medium (bacto-peptone (Difco): 3.0 g/l, yeast extract (Difco): 2.0 g/l, glucose: 20 g/l, K_2HPO_4: 1.0 g/l, $MgSO_4 \cdot 7H_2O$: 5.0 mg/l, CA (Kishida, DS1.5) 1000 mg/l) at 28 °C and 120 rpm on a rotary shaker. The plastic film and 5 ml of each bacterial culture at mid-log phase were added to 100 ml of fresh medium in a 300-ml Erlenmeyer flask, and incubated at 28 °C and 120 rpm on a rotary shaker.

5.2.5. Degradation of CA plastic film by enzymes

To a 300-ml Erlenmeyer flask was added a CA plastic film and 100 ml of the CA-degrading crude enzyme solution in phosphate buffer (pH 7.0), which was prepared from 1000 ml of the culture supernatant of strain S2055. The flask was incubated at 30 °C and 120 rpm on a rotary shaker. For the degradation tests, pH of the reaction mixture was adjusted at 7.5. Similar degradation tests were carried out with commercially-available cellulase, esterase and/or lipases solely or in combinations instead of the crude enzyme. In these cases 50 units of enzymes were suspended into 100 ml of the phosphate buffer. During the incubation, the weight loss of the plastic film was periodically measured in duplicate experiments. All standard errors were less than 2.5 % and the results showed good reproducibility.

5.2.6. Enzyme assays

Cellulase activity was measured by the methods of Thomas *et al.* (1988). Portions of

enzyme solution (5 ml) was added to 5 ml of 50 mM phosphate buffer (pH 5.6) containing 1.0 % of cellulose powder (Advantec C, Toyo Roshi Kaisya, Ltd., Tokyo, Japan). After incubation at 40 °C for 120 min, the amount of released reducing sugar was measured by the Somogyi-Nelson method (Somogyi 1952), using D-glucose as a standard. Lipase activity was measured by method of Iwai *et al.* (1969). Portion of enzyme solution (1 ml) was added to 9 ml of 100 Mm of acetate buffer (pH 4.8) containing 20 % of olive oil (Wako, Osaka, Japan). After incubation at 30 °C for 60 min, amounts of released fatty acids were measured by Alkali titration method (Alford and Pierce 1963). Esterase activity was measured by method of Pocker and Stone (1967). Portion of enzyme solution (1 ml) was added to 9 ml of 50 mM of Tris-HCl buffer (pH 8.0) containing 1.0 % of *p*-nitrophenyl acetate (Wako, Osaka, Japan). After incubation at 30 °C for 15 min, amounts of released *p*-nitrophenol were measured as absorbance at 450 nm. For comparing the substrate specificity of the lipases in the crude enzyme of strain S2055 and commercially-available lipases, tri-n-orein, tri-n-butyrin, tri-n-acetin, methyl-n-butyrate, Tween 80 and olive oil were used instead of olive oil, and the same assays were performed.

5.2.7. Determination of weight loss of CA plastic film

Weight measurement of CA plastic was carried out by procedure referred to 4.2.7. In preliminary tests, the proportionality between the wet weight (measured by this method) and dry weight of the plastic film in the degradation process was confirmed (proportional value was 0.68 and correlation coefficient was 0.991).

5.2.8. Deacetylation of CA by enzymes

CA with DS 1.5 was applied to the deacetylation tests by 3 major protein fractions of the

crude enzyme and commercially-available enzymes. The reaction mixture contained 100 ml of the enzyme solution and 900 ml of acetate buffer (pH 4.8) containing 2 % of CA. After the incubation at 28 °C for 5 days, deacetylability of CA was estimated by thin layer chromatography (TLC) with silica plates according to Kamide *et al.* (1982).

5.3. RESULTS AND DISCUSSION

5.3.1. Natural abundance and variety of CA-degrading bacteria

In order to evaluate the CA-degrading potentials and to screen the CA-degrading bacterium existing in the natural or semi-natural environment, CA-degrading bacteria in various soil and water samples (5 forest soils, 7 field soils, 3 activated sludges and 4 landfill leachates and a sediment) were enumerated addition to Chapter 2. Table 5.1 summarizes the counts of CA-degrading bacteria in comparison with those of heterotrophic bacteria. The population of CA-degrading bacteria accounted for 0.02 to 26 % of the heterotrophic bacterial populations as

Table 5.1. Natural abundance of CA degraders and heterotroph in various environments. Viable counts of bacteria are shown as cfu/ml-water or g-soil.

Sample	heterotrophic bacteria	CA-degrading bacteria
Forest soil-1	1.4×10^6	1.5×10^5 (11) [a]
Forest soil-2	1.2×10^8	3.4×10^4 (0.03)
Forest soil-3	1.9×10^6	5.0×10^5 (26)
Forest soil-4	2.4×10^6	1.0×10^5 (4.2)
Forest soil-5	8.3×10^7	4.1×10^5 (0.5)
Field soil-1	2.2×10^7	3.0×10^6 (14)
Field soil-2	2.1×10^7	1.0×10^5 (0.5)
Field soil-3	6.4×10^7	9.6×10^5 (1.5)
Field soil-4	3.3×10^8	3.2×10^5 (1.0)
Field soil-5	3.0×10^6	3.5×10^4 (1.2)
Field soil-6	9.1×10^7	2.9×10^5 (1.5)
Field soil-7	1.0×10^9	6.6×10^5 (0.07)
Activated sludge-1	1.8×10^9	9.0×10^5 (0.5)
Activated sludge-2	3.0×10^9	8.5×10^7 (2.8)
Activated sludge-3	2.1×10^9	1.0×10^8 (4.8)
Landfill leachate-1	1.0×10^5	1.0×10^2 (0.1)
Landfill leachate -2	1.5×10^5	2.7×10^4 (18)
Landfill leachate -3	6.5×10^4	6.7×10^1 (0.1)
Landfill leachate -4	8.8×10^4	3.5×10^2 (0.4)
Landfill sediment-1	3.1×10^5	1.0×10^3 (0.3)

[a] Values in parenthesis indicate the ratios of cfu of CA-degrading bacteria to that of heterotrophic bacteria.

halo-forming cfus. And they existed at the population sizes between 6.7 x 10^1 and 1.0 x 10^8 cfu/g-soil or ml-water. Although the population size varied depending on the samples, it seems that CA-degrading bacteria commonly exist in the natural environment.

It is also important to know what kinds of CA-degrading bacteria exist in the natural environment as well as their numbers. Morphologically-different 35 colonies which showed clear halos were isolated from the emulsified-CA plates, and taxonomically identified. Table 5.2 shows the results of classification of the isolates. 35 isolates were classified into 15 genera, including both gram-positive and gram-negative groups. Until now little effort has been done to clarify the taxonomy of CA-degrading bacteria or microbes, consequently, only 3 CA-degrading bacterial strains have been reported with clear taxonomical identification, i.e., a *P. paucimobilis* (Nelson *et al.* 1993) and 2 strains of *N. sicca* (Sakai *et al.* 1996). The results of the bacterial classification shown here suggested that the CA-degrading potential distribute in a relatively wide variety of bacteria.

Table 5.2. Dominant bacterial genera isolated from various environmental samples as CA degrading bacteria

(Gram-positive)	*Corynebacterium*	5
	Listeria	1
	Clostridium	2
	Bacillus	4
(Gram-negative)	*Eikenella*	1
	Acinetobacter	1
	Moraxella	1
	Flavobacterium	3
	Pseudomonas	3
	Xanthomons	2
	Cardiobacterium	3
	Achromobacter	1
	Haemophilus	5
	Campylobacter	1
	Streptobacillus	2
Total		35 strains

Based on these results, it appears that CA has a good possibility for biodegradation in various environment. Thus, it is important to understand the detailed mechanism(s) of CA degradation by these bacterial strains for finding out the key factor(s) to enhance the biodegradability of CA plastics in the environment and the waste disposal processes.

Degradation of CA plastic film by CA-degrading bacteria

Table 5.3 shows the results of the degradation tests of CA plastic film by the isolated CA-degrading bacteria which showed relatively-large halos on the emulsified-CA medium. All 19 bacterial strains used here could be grown on CA-BSM repeatedly, therefore, it may be said that they are able to utilize powdery CA with DS 1.5 for their growth. As shown in the table, most of the isolates showed degradation of CA plastic film (DS 1.7) to a certain extent; the control experiments performed without the bacterial inoculation showed no significant weight loss of the CA plastic film. However, when CA-plastic film with DS 2.5 was used, all the isolates showed insignificant or a trivial weight loss, less than 3 %. It is generally believed and observed

Table 5.3. Biodegradability of CA films by CA-degrading bacteria.

source	Strain	DS1.5	DS2.5
forest soil	Nn01	+	–
	Nn02	+	+
	Nn03	+	+
	Nn04	+	+
	Nn05	–	–
	Nn06	+	–
field soil	Ns01	+	+
	Ns02	+	–
	Ns03	+	–
	Ns04	+	–
	Ns05	+	–
	Ns06	+	–
	Ns07	+	–
landfill	S2051	–	–
	S2052	+	–
	S2053	+	–
	S2054	+	–
	S2055	++	+
	S4050	–	–

++: weight loss more than 10%, +: 0 to 10%, -: no weight loss

that CA with higher DS values are more recalcitrant against biodegradation (Nelson *et al.* 1993, Sakai *et al.* 1998). In this study, colonies showed halo formation against emulsified-CA (DS 3.0) were isolated as CA-degrading bacteria. However, none of the tested isolates could significantly degrade CA plastic film (DS 2.5) and even CA plastic film (DS 1.7) was not degraded by a few isolates. These paradoxical results suggest that the biodegradability of CA is strongly influenced not only by the DS value but also by its form, e.g. water-soluble, emulsified, powdered or film-formed etc. It is also possible that the halo formation on the emulsified-CA medium was not due to real degradation of the CA with DS 3.0 but due to partial degradation of the less substituted parts in the polymer molecule.

Among the isolates tested, only strain S2055 which was isolated from a waste landfill leachate showed more than 10% (11.1 %) of the weight loss for the CA plastic film. Plating of this strain resulted in the formation of larger clear halos along with the colonies, suggesting higher capability for CA degradation compared with the other isolates. Therefore, strain S2055 was used for further studies. Morphological and physiological properties of strain S2055 were described in Fig. 5.1 and Table 5.4. From these results, strain S2055 was identified as *Bacillus* sp. The time course of the degradation of CA plastic film by this strain is shown in Fig. 5.2. The weight loss reached 10 % after 10 days, however, it did not proceed only a little even after 5 weeks.

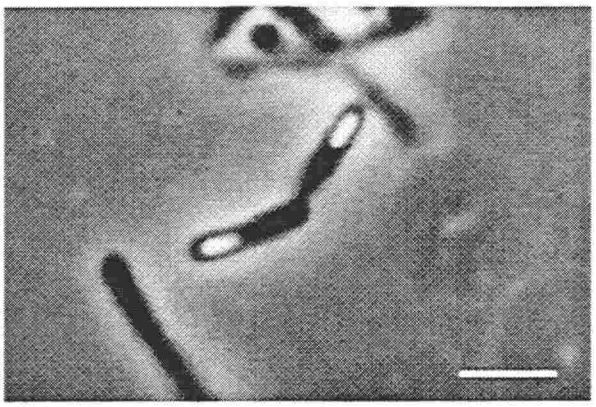

Fig. 5.1. Phase contrast microscopic observation of strain S2055. Bar indicates 5 μm.

Table 5.4. Morphological and physiological properties of *Bacillus* sp. S2055

Characteristic	
Form	Rod
Sizes	0.7-1.0 x 5-10μm
Gram stain	Positive
Spores	Forming
Motility	+
Anaerobic growth	+
Catalase	+
Oxidase	–
Reduction of NO_3	+
Indole	–
Urease	–
Gelatinase	+
Acid production from	
Glucose	+
Sucrose	+
Inositol	–

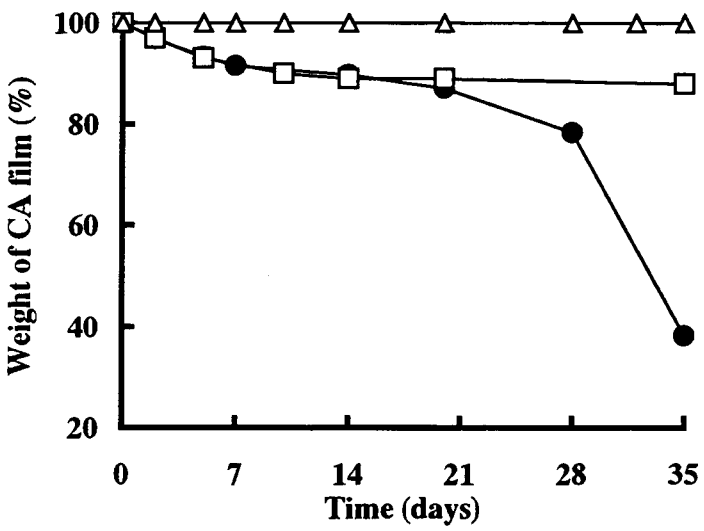

Fig. 5.2 Weight loss of CA plastic film degraded by strain S2055 and crude degrading enzyme extracted from S2055. △: control, □: S2055, ●: crude enzyme.

5.3.2. Degradation of CA plastic film by the crude enzyme from strain S2055

Degradation of the CA plastic film by the crude enzyme extracted from the culture supernatant of strain S2055 is shown in Fig. 5.2 in comparison with that by the growing bacterial culture. The weight loss of the CA by the enzyme solution reached 62 % during 5-

week exposure. As shown in Fig. 5.2, the course of CA plastic degradation by the crude enzyme was divided into two phases; the gradual weight loss for initial 20 days (lag phase) and the following phase showing the rapid weight loss (weight loss phase). Apparent deterioration of the CA film started from day 20, that is the transition point from the lag phase to the weight loss phase, and it was fragmented into small pieces afterwards (Fig. 5.3), although such fragmentation was not observed at all by the bacterial cultures. Thus, the application of the enzyme solution led to much more efficient degradation of the CA plastic than that of the bacterial culture. Similar observation was previously reported for degradation of a PVA-starch blend plastic film, and possible reasons why the application of enzyme solution caused superior results have been already discussed in Chapter 4.

Sakai *et al.* (1996) proposed that CA biodegradation is mediated by the cooperative reactions of esterase(s) (or lipase(s)) and cellulase(s). This is very likely when considering the structure of CA. As to estimate the enzymatic system in strain S2055 which is responsible for the CA degradation, activities of the above-mentioned enzymes were assayed in the culture broth of strain S2055. Fig. 5.4 shows the typical courses of cell growth and enzyme production of strain S2055 in the CA-BSM medium. Though esterase activity was not detected throughout

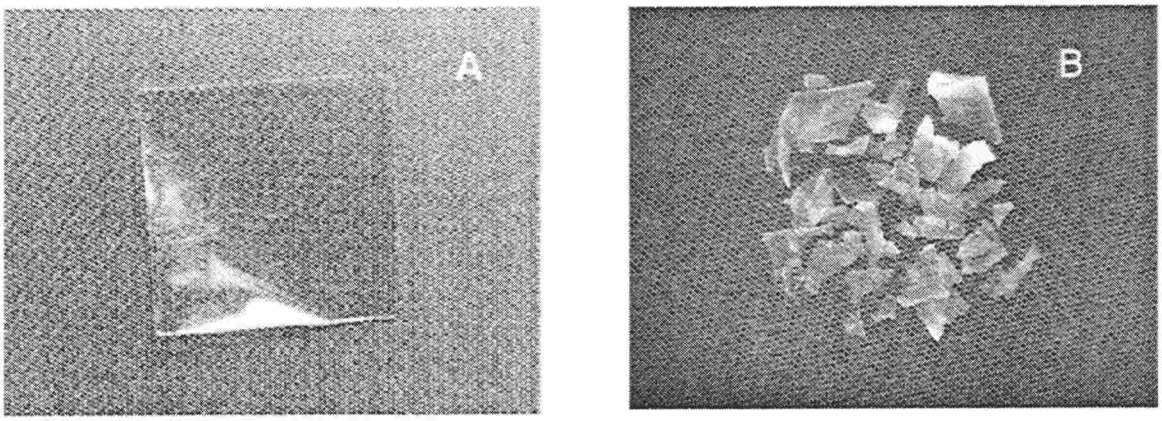

Fig. 5.3 Pictures of CA plastic films. (A) before the degradation test and (B) after the 5-week exposure to crude enzyme solution

the cultivation period, lipase and cellulase activities were detected in the culture supernatant. Judging from that higher enzymatic activities were found in the culture supernatant than in the cells, these enzymes are extracellular (data not shown). Activities of lipase and cellulase in the culture supernatant exhibited different patterns of time courses. Both enzymatic activities increased rapidly during the logarithmic growth, however, the lipase activity showed a sudden and considerable drop after that, whereas the cellulase activity was maintained stable during 10-day cultivation. Patterns of the production of these enzymes in CA-GPY medium were similar to those in the CA-BSM. There was not any difference when the media was prepared without the CA (DS 1.5) and in the presence or absence of the CA plastic film, indicating CA-independent production of the enzymes.

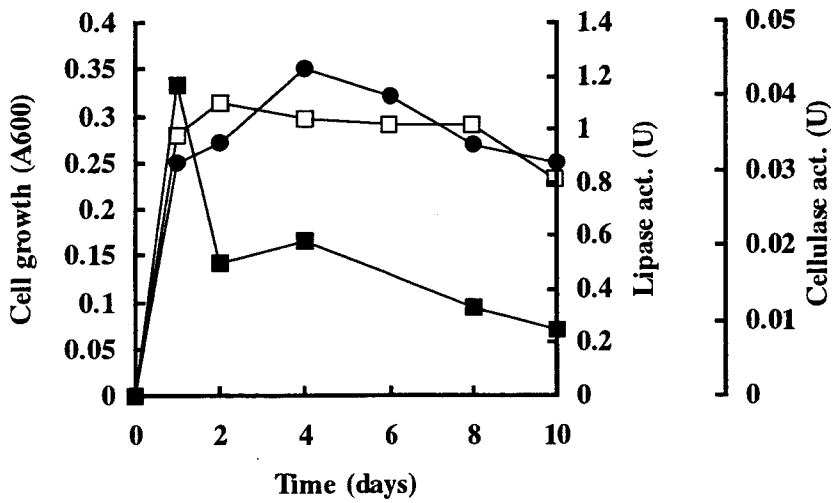

Fig. 5.4. Cell growth and enzymatic activity of strain S2055 in CA-BSM media.
●: cell growth, ■: lipase activity, □cellulase activity.

Table 5.5. Comparison of enzymatic degradation of CA plastic after 35 days.

Applied enzyme	Weight loss (%)
crude enzyme from S2055	61.9
cellulase from *Aspergillus niger* *1	19.5
lipase from *Rhizopus arrhizus* *2	8.1
esterase from porcine liver	7.9
lipase and cellulase (*1, *2)	18.7
control	0

Commercially-available, known or typical cellulase, lipase and esterase were applied to the degradation tests of the CA plastic film in the same manner as the crude enzyme for comparison (Table 5.5). The weight loss of the CA film exposed to the commercial cellulase was less than 20 % in 5 weeks, while that by the crude enzyme was 62 %. Although the commercial lipase or esterase application showed weakly positive results (both approximately 8 % of weight loss), combination of the cellulase and lipase could not enhance the weight loss and did not resulted in the film fragmentation. These results suggested that the cellulase(s) and/or lipase(s) produced by strain S2055 possess unique substrate specificities which may be different from well-known enzymes.

5.3.3. Partial characterization of the CA-degrading enzyme(s) of strain S2055

For characterizing the CA-degrading enzyme(s) in strain S2055, the crude enzyme preparation was subjected to the fractionation by GPC. The GPC chromatogram showed 3 major protein peaks (Fig. 5.5A). The mean molecular mass of these protein fractions named I, II and III were estimated at approximately 800, 75 and 20 kDa, respectively. As shown in Fig. 5.5B, all the protein peaks, Fractions I-III, corresponded to relative high lipase peaks. On the other hand, esterase activity was not detected from any fractions as well as the culture supernatant. Furthermore the crude enzyme was confirmed to contain the lipase-like activity rather than esterase-like activity according to the method of Laws and Moore (1963) that compare the deacetibility of dissolved and emulsified tri-n-acetin. Cellulase activity was found only in the largest-Mw fraction (Fraction I) with a quite high molecular mass as a single protein or enzyme. The previous paper reported that cellulases generally form cellulosomes (Lamed *et al.* 1983), which are greatly-associated complexities of enzymes and cellulose binding proteins. It was reported that many non-cellulase enzymes such as xylanase, hemicellulase and lichenase were

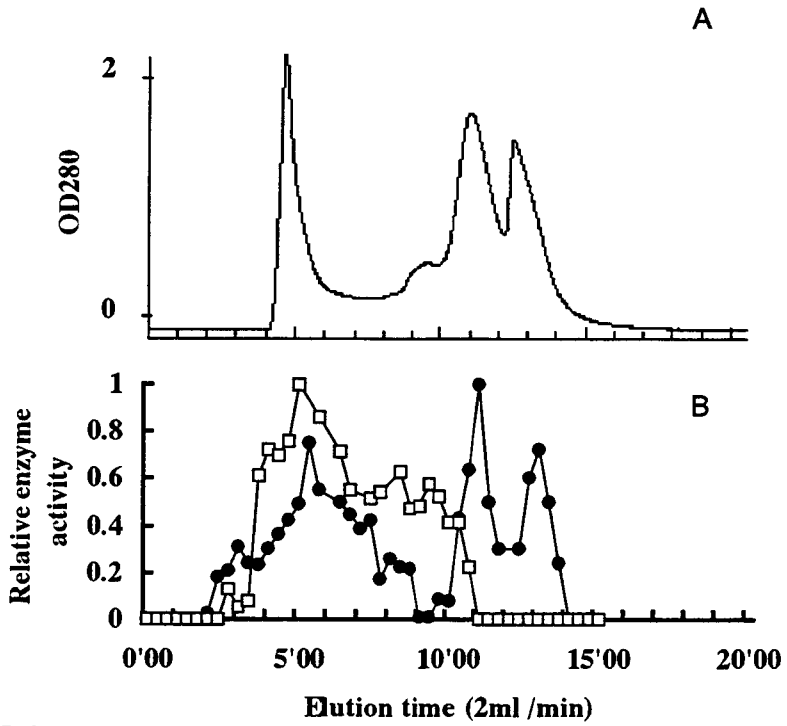

Fig. 5.5. Separation of CA degrading crude enzyme and their cellulase and lipase activities. (A) Chromatogram for protein concentration (B) Specific cellulase and lipase activities of the each fractions. □: cellulase activity ◆: lipase activity

included in a cellulosome (Kataeva *et al.* 1999), and sometimes their molecular mass would be over 10-MDa (Gal *et al.* 1997, Salamitou *et al.* 1994). The Fraction I was also supposed to form a cellulosome-like structure including both cellulase and lipase-like activities.

To identify which lipases are active against CA, deacetylating activity was investigated with these Fragments I-III. Commercially-available enzymes were also used for comparison. Table 5.6 shows the results of the CA deacetylation tests. Of the 3 fractions, only Fraction I showed significant deacetylation against the CA (DS 1.5), while the other fractions and commercially-available enzymes did not. When the CA plastic degradation tests were carried out using Fractions I-III in a smaller scale, only Fraction I could degrade the film into pieces, though the film fragmentation did not occur with the other fractions even in the presence of the commercially-available cellulase (data not shown). These results strongly suggested that the lipase found in Fraction I played the key role in the CA plastic degradation. In other words, the

deacetylation mediated by this lipase would be necessary for subsequent hydrolysis of the main chain of the CA. Although Sakai *et al.* (1996) suggested almost the same mechanism, there was no clear description about the key enzyme (lipase or esterase).

Further, lipase-like activity in the fraction I-III against various substrates were investigated, and compared with previously reported lipases (Table 5.7). Fractions I-III showed different substrate availability, as well as previously-reported multiform-lipases (Semeriva *et al.* 1967).

Table 5.6. Deacetylation of CA with DS 1.5 by various commercial enzymes and fraction I – III.R_f and DS value estimated by TLC assay were shown.

Applied enzyme	R_f	Estimated DS
Fraction I	0.63	0.82
Fraction II	0.36	1.53
Fraction III	0.37	1.50
lipase (A)	0.38	1.48
lipase (B)	0.37	1.50
lipase (C)	0.36	1.53
cellulase from *Aspergillus niger*	0.38	1.48
lipase (A) and cellulase	0.38	1.48
control	0.37	1.50

A: from *Rhizopus arrhizus*, B: from *Pseudomonas*.sp., C: from *Candida rugosa*

Table 5.7. Deacetylability against several substrates of fractions I – III and previously reported lipase as a comparison. Activities were measured in the 50mM acetate buffer (pH 5.6) at 28C for 24h. Relative activities were shown as a percentage of deacetylation activity toward tori-n-orein or olive oil.

	tri-n-orein	tri-acetin	tri-n-butyrin	methyl-n-butyrate	tween 80	Olive oil
Fraction I	100	10.0	93.3	160	0	143
Fraction II	100	35.9	332	25.0	6.5	204
Fraction III	100	5.5	2.8	2.7	0	119
*Penicillium cyclopium**	100	48.6	228	6.9	4.3	–
*Geotrichum candidum**	100	0	3.6	1.6	0	–
*Aspergillus niger**	100	30.3	54.3	0	9.8	–
Rhizopus delemar A*	100	29.9	101	2.7	2.1	–
Rhizopus delemar C*	100	0	104	2.6	1.7	–
*Chromobacterium***	100	–	137	–	3.78	222
Pseudomonas sp.**	100	–	377	–	4.5	80.6
*Candida rugosa***	100	–	171	–	9.2	447
Rhizopus sp.**	100	–	6170	–	11.7	27500
*Aspergillus usamii***	0	–	220	–	0	100
Bacillus sp.***	100	42	98	2	6	–
*B. thermocatenulatus*****	100	–	–	–	–	115
*B. thermoleovorans******	100	–	7.1	–	4.7	40.8

reffered to * Tsujisaka *et al.* (1972), ** Plou *et al.* (1998), *** Sugihara *et al.* (1991), ****Schmidt-Dannert *et al.* (1994),***** Lee *et al.* (1999)

Fractions II and III exhibited substrate specificity profiles similar to those of previously-reported lipases derived from *Penicillum* sp. and *Geotrichum* sp. (Tsujisaka *et al.* 1972), respectively. On the other hand, Fraction I showed very different substrate availability from that of any other fractions or previously-reported lipases (Tsujisaka *et al.* 1972, Plou *et al.* 1998, Sugihara *et al.* 1991, Schmidt-Dannert *et al.* 1994, Lee *et al.* 1999) as far as we investigated. Although the crude enzyme was distinguished as lipase from esterase according to Laws and Moore (1963), it was still difficult to clearly divide the esterase and lipase because of their crude purification. However the unique lipase-like activity of Fraction I suggests that CA specificity may not be a common property of lipases and such an unpublished lipase would play a key role in the CA degradation or deacetylation.

Since many reports including the study indicated that the existence of substituted unit can inhibits the degradation of cellulose derivatives by cellulase (Bhattacharjee and Perlin 1971, Reese 1957), further detailed studies on such a peculiar CA-deacetylating enzyme might be important. The highest DS which can be deacetylated by the lipase in Fraction I should be determined, for example. Moreover, it seems necessary to screen other particular lipases capable of deacetylating wide ranges of CA plastics with different DS and/or Mws.

5.5. REFERENCES

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Chapter 6
Summary and Conclusion

Although biodegradable plastics has become a public concern as a countermeasure for the serious plastic waste problems, the degradability in waste landfill site has been not well characterized. Therefore, this study focused on enhancement of *in situ* plastic degradation using the biostimulation technique and enzymatic degradation of plastic was examined as the bioaugmentation or pretreatment degradation process.

First, the population sizes of PDMs in a landfill site were estimated in order to assess the *in situ* plastic degradation potentials (Chapter 2). PDMs for each polymers ubiquitously and abundantly presented in the landfill sites. Although PDMs for natural polymers, PHB and CA, were found from all the samples, those of chemically-synthesized polymers, PCL, PLA and PEG, could not be always detected. Further, PDMs for natural polymers accounted for higher percentages to the heterotrophic bacteria than PDMs for chemically-synthesized polymers as a whole. This suggests that the degradation potential of plastics composed of chemically-synthesized polymers in the natural environments including landfill sites is inferior compared to that of plastics composed of natural polymers. It may be concluded that certain degradation potentials of various kinds of biodegradable plastics exist in the landfill sites, and that the proper management or operation of the environmental conditions, e.g. mechanical aeration of the leachate, will lead to stimulation of PDMs.

In order to stimulate indigenous PDMs and to enhance the *in situ* degradation of plastics, application of bioventing to waste landfill was examined. The fates of commercial biodegradable plastic films, in the model landfill reactor, were pursued with and without bioventing (Chapter3). As a result, the application of bioventing to the landfill could considerably stimulate the growth and activity of certain groups of PDMs (e.g. PDMs for PHB, starch and lower-substituted CA) and, consequently, could enhance the degradation of certain biodegradable plastics. When the bioventing is applied to the landfill coupled with the use of the PHB plastic, more rapid or efficient settlement of the landfill might be achieved. On the other hand, bioventing may not contribute much to accelerate the volume reduction of starch-PVA blend and CA plastics, because the fragmentation or complete degradation of these plastic films did not occur even in the BVL reactors. To achieve the fragmentation of these plastics, stimulation of the microbial population which can degrade recalcitrant parts of the plastics, i.e. PVA and highly-substituted CA, would be requisite.

Therefore, specific degrading microbes and enzymes were applied to plastics which were not effectively degraded even in the BVL reactor. Application of the PVA-degrading bacterium and/or enzyme to starch-PVA blend plastic resulted in a maximal weight loss of about 70% and film breakage though this strain did not show any starch-degrading activity (Chapter 4). Especially, enzyme application led to the film fragmentation into small pieces within 10 days. On the other hand, application of starch-degrading bacterium and α-amylase did not effectively occur the effective degradation and fragmentation of films. It was indicated that degradation of the PVA fraction is important and requisite for complete degradation or decomposition of this plastic film. In Chapter 5, enhanced CA degradation by degrading bacterium and its enzyme was demonstrated. By applying the CA-degrading crude enzyme produced by a novel CA-degrading bacterium *Bacillus* sp., a weight loss reached to 62 % and film was rapidly and totally

fragmented. This crude enzyme had three different Mw fractions. Amongst them, only the largest Mw fraction showed significant deacetylation against the CA. The unique substrate specificity as lipase of this fraction suggests that capability of CA degradation may not be a common property of lipases and such an unpublished lipase would play a key role in the CA degradation or deacetylation. Based on these results, the enhanced degradation of these plastics would need the peculiar degrading microbes or enzymes. Since such microbial populations are considered to exist rarely in the landfill site, their bioaugmentation seems necessary to achieve the enhanced in-situ degradation of these plastics.

Recently there have been much concern regarding the serious problem about municipal or hazardous waste treatment. Landfill sites are thought to be dangerous and troublesome areas because their stabilization would take a long time. Especially plastic wastes are one of the most problematic materials owing to their persistence against the biodegradation. From the results of this study, it was indicated that biodegradable plastics, which has been proposed as a solution for the problems of plastic wastes, are not always degraded enough in the landfill sites. Best opportunities for the utilization of biodegradable plastic to address solid waste issues are found in emerging managed landfill i.e. application of bioventing to landfill. However, fragmentation or complete degradation of some plastics cannot be achieved even in such a managed landfill, suggested that degradation of the recalcitrant part of the plastics would be requisite. Since such microbial populations rarely exist in the landfill sites, augmentation of microbes or enzymes would effectively achieve the enhanced *in-situ* degradation of these plastics.

The biostimulation technique would be applicable to the waste landfill on reference to the previous bioremediation projects, which provide a lot of aspects on designing and operating the process. In this study, certain effect of biostimulation for the improvement of the *in-situ* plastic degradation was confirmed. However biostimulation process might affect the behavior of other

component in the waste such as organo-chemical pollutants or heavy metals. Therefore further studies would be needed for developing the biostimulation process in the waste landfill. On the other hand, bioaugmentation has been often researched but rarely implemented successfully even in the laboratory scale experiment. There are numerous reasons for difficulties in the use of introduced bacteria, i.e. growth inhibition of introduced bacteria caused by competition with indigenous bacteria. That is, many difficulties are still remaining for the practical application of bioaugmentation. Solution of these problems and proper use of biodegradable plastic will lead the effective reduction of plastic waste in the waste disposal process.