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Distribution of Poly(β-hydroxybutyrate) and Poly(ϵ-caprolactone)Aerobic Degrading Microorganisms in Different Environments

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To assess the capacity of the natural environment for degrading plastics, the populations of poly(β-hydroxybutyrate)(PHB)- and poly(ϵ-caprolactone)(PCL)-degrading aerobic microorganisms and their ratios to the total number of microorganisms in soil samples were estimated by the plate count method with agar medium containing emulsified PHB or PCL. The numbers of the degrading microorganisms were determined by counting colonies that formed clear zones on the plate. It was found that PHB- and PCL-degrading (depolymerizing) microorganisms are distributed over many kinds of material, including landfill leachate, compost, sewage sludge, forest soil, farm soil, paddy soil, weed field soil, roadside sand, and pond sediment. Of total colony counts, the percentages of PHB and PCL degrading microorganisms were 0.2–11.4 and 0.8–11.0%, respectively. The results suggest that many kinds of degrading microorganisms are present in each environment and that specific consortia differing in biodegradation capacity are constructed.

KEY WORDS: Biodegradable plastic; poly(β-hydroxybutyrate); poly(ϵ-caprolactone); aerobic degrading microorganisms; distribution; biodegradation capacity.

INTRODUCTION

The study of the biodegradation of plastics is important for solving plastics disposal problems.

Many reports on the synthesis and degradation mechanisms of biodegradable plastics have been published. However, ecological investigations concerning the influence of the biodegradation of plastics on the environment have not been conducted. Only a few genera and species of plastic degrading microorganisms have been reported. For example, only 6 genera and 14 species of microorganisms have been reported as degrading PHB by extracellular enzyme systems [1–11]. The number of reports on the ecological behavior of plastic degrading microorganisms is also limited. To prevent new environmental problems caused by the biodegradation of plastics, it is important to learn as much as possible about the capacity of the natural environment to degrade plastics, the effects of the biodegradation products, and the role of plastics-degrading microorganisms.

To assess the capacity of the natural environment for degrading plastics, all degrading microorganisms in each environment, including microorganisms having slower propagation times and lower degradation activity, should be enumerated and the percentages of the plastics-degrading microorganisms to the total number of microorganisms present should be determined. The plate count method is commonly used for enumerating microorganisms in the environment [12–19]. The clear-zone method was used by Chowdhury [1], Delafield *et al.* [2], and Gilmore *et al.* [20] to confirm the isolation of poly(β-hydroxybutyrate) (PHB)-degrading microorganisms. By using the plate count and clear-zone methods together, it is possible to count the degrading microorganisms and obtain their percentage to the total population.

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A method of counting PHB- and poly(ϵ-caprolactone) (PCL)-degrading (depolymerizing) microorganisms was investigated. The populations of the PHB- and PCL-degrading aerobic microorganisms and their ratios to the total number of microorganisms in different environments were estimated by the plate count method with agar medium containing emulsified PHB or PCL. The numbers of the degrading microorganisms were estimated by counting colonies that formed clear zones on the plate. Distribution of PHB- and PCL-degrading microorganisms and the biodegradation capacity of the environment are discussed.

EXPERIMENTAL

Materials

PHB [BX GV9(EE) additive-free, technical-grade granules] from *Alcaligenes* sp. was obtained from ICI (Japan) Limited. This was dissolved in hot chloroform and purified by precipitation with hexane. The weight-average molecular weight of the PHB was estimated to be about 2.2×10^5 from intrinsic viscosity data according to the method of Einaga *et al.* [21]. PCL (TONE P-767; number-average molecular weight, 40,000) was obtained from Union Carbide Corporation.

Agar Plates Containing Emulsified PHB or PCL

Agar plates containing suspended PHB were used for cultivation of PHB degrading bacteria and for enzymatic assays by Chowdhury [1], Delafield *et al.* [2], and Gilmore *et al.* [20]. In these previous investigations, the agar plates were prepared using PHB granules isolated from PHB-producing bacteria or ground PHB powder.

In cases of other polymers unavailable as granules, the agar plates have to be prepared using ground powder, suspension or emulsion. For counting colonies and measuring clear zones formed on plates, the polymer should be finely dispersed into the medium with maximum homogeneity and appropriate turbidity.

Desired agar plates containing emulsified high molecular weight PHB or PCL were prepared by one emulsification method with a homogenizer. Two agar plates containing a polymer, one poor in nutrients (yeast extract, 250 mg/liter) (YE-plate) and one rich in nutrients (nutrient broth, 8000 mg/liter) (NB-plate), were prepared.

PHB (1.00–1.59 g) or PCL (1.00 g) was dissolved in 40 ml of methylene chloride. Each solution was emulsified with a homogenizer into 1000 ml of a basal medium composed of 250 mg/liter yeast extract, 10 mg/liter $FeSO_4 \cdot 7H_2O$, 200 mg/liter $MgSO_4 \cdot 7H_2O$, 1000 mg/liter $(NH_4)_2SO_4$, 20 mg/liter $CaCl_2 \cdot 2H_2O$, 100 mg/liter NaCl, 0.5 mg/liter $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg/liter Na_2WO_4, 0.5 mg/liter $MnSO_4$, and 100 mg/liter surface-active agent Plysurf A 210G [Daiichi Kogyo Seiyaku; $RO(CH_2CH_2O)_nP(=O)(OH)OR'$; R, alkyl or alkylallyl group; R', $-H$ or $-(CH_2CH_2O)_nR$; hydrophile-lipophile balance, 9.6] in 10.7 m*M* KH_2PO_4/K_2HPO_4 (pH 7.1). The PHB solution was also emulsified into a medium (pH 7.0) composed of 8000 mg/liter nutrient broth. Agar (1.5%, w/v) was added to the emulsified medium and dissolved by heating, which simultaneously evaporated methylene chloride from the medium. The agar medium was autoclaved at 120°C for 20 min and then poured into petri dishes.

This preparation method of the polymer-containing agar medium is adaptable for any polymer that can be emulsified into an agar matrix.

Soil Samples

Samples from 10 locations were collected on 28 January 1992 from different environments at Tsukuba and environs: landfill leachate (Nos. 1 and 2 from Tokyo Bay), sewage sludge compost (No. 3 from Tokorozawa in Saitama prefecture), sewage sludge supernatant (No. 4), forest soil (No. 5), farm soil (No. 6), paddy soil (No. 7), weed field soil (No. 8), roadside sand (No. 9), and pond sediment (No. 10). The water contents were calculated from weight loss of the samples after drying at 105°C for 24 h. The organic contents were calculated from the weight loss of the dried samples after pyrolysis at 600°C for 18 h. The residue was regarded as inorganic substance.

Culture

Each soil sample was diluted 10–10^4 fold with sterilized basal medium (pH 7.0). A 0.1-ml aliquot of each diluted sample was spread onto an agar medium plate containing emulsified PHB or PCL. The cultures were incubated at 30°C. Visible colonies and clear zones that formed on the plates were counted almost every day of the culture period (up to 10–30 days). The degrading microorganisms were isolated from colonies that formed clear zones on the plates.

PHB Degradation Tests

Two test tubes containing 10 ml of basal medium and 20 mg of fibrous PHB, which was purified by reprecipitation, were inoculated, one with 0.1 ml of a first enrichment broth from a soil sample (cultured at 30°C

for 74 h) and the other with a colony from a plate. The test tubes were aerated by stirring with a reciprocal shaker at 400 strokes/min and 30°C. After 45 h (or 93.5 h) and 46 h, respectively, the media were filtered through Toyo No. 2 qualitative filter paper. The remaining PHB was collected and washed with water. The collected residual PHB was weighed after drying at 105°C for 20 h.

RESULTS AND DISCUSSION

Formation of Colonies and Clear Zones

Ten samples collected from different environments were used in this study. Each sample differed in content of water, organic material, and inorganic substances (Table I).

Table I. Composition of Samples

Soil sample	Composition (wt%)		
	Water	Organic substances	Inorganic substances
1. Landfill leachate (No. 1)	99.62	0.08	0.31
2. Landfill leachate (No. 2)	99.08	0.16	0.76
3. Sewage sludge compost	33.25	26.48	40.27
4. Sewage sludge supernatant	99.80	0.12	0.08
5. Forest soil	53.28	19.55	27.17
6. Farm soil	43.65	11.29	45.05
7. Paddy field soil	52.79	6.59	40.62
8. Weed field soil	40.54	9.13	50.33
9. Roadside sand	2.10	6.25	91.65
10. Pond sediment	97.41	0.30	2.29

After 1–13 days of colony formation on the agar plates, a circular clear zone formed around each colony containing a microorganism capable of depolymerizing the polymer (Fig. 1). The clear zones on a plate varied

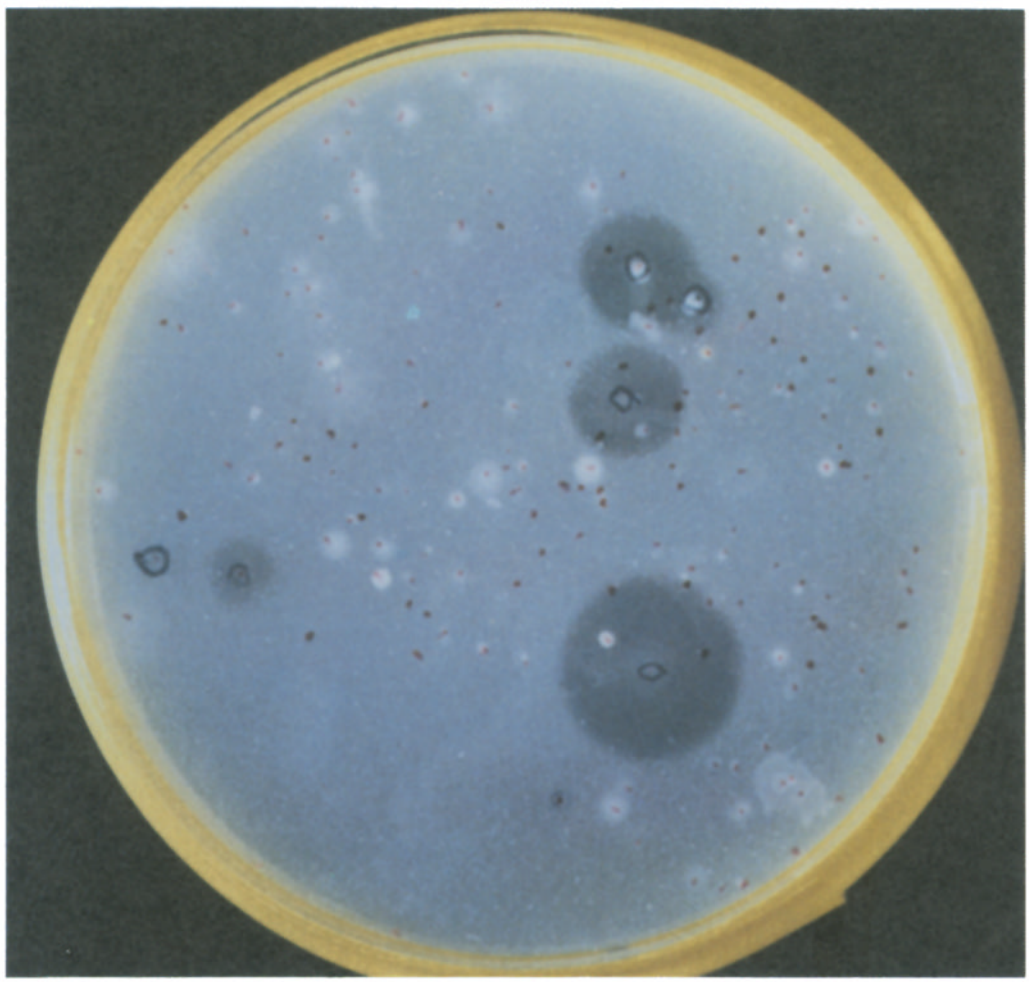

Fig. 1. Colonies and clear zones on a YE-plate containing emulsified PHB. Sample was landfill leachate; culture at 30°C for 6 days.

in size and clarity. Usually, a distinct clear zone was found around each different colony in morphology. The size and clarity of the clear zone apparently reflect the physiological state of the PHB-degrading microorganisms, diffusion rates of different enzymes in agar medium, and amount and activity of enzymes. Heterogeneity in the size and clarity of the clear zones on the plates may indicate the presence of a mixed population of degrading microorganisms.

The numbers of visible colonies and clear zones were counted at specified times and colony and clear-zone formation curves (CCFCs) were drawn. The colony formation curve (CFC) proposed by Ishikuri and Hattori [12] represents the time required for formation of visible colonies. The numbers of colonies and clear zones were expressed as colony- or clear zone-forming units (CFU) per 1 g of soil sample. Typical CCFCs on a YE-plate containing emulsified PHB are shown in Fig. 2 (Sample No. 1, landfill leachate). The numbers of colonies and clear zones on YE-plates containing PHB were counted for 30 days. Both were found to increase stepwise. The CCFCs in Fig. 2 are in tiers, indicating that

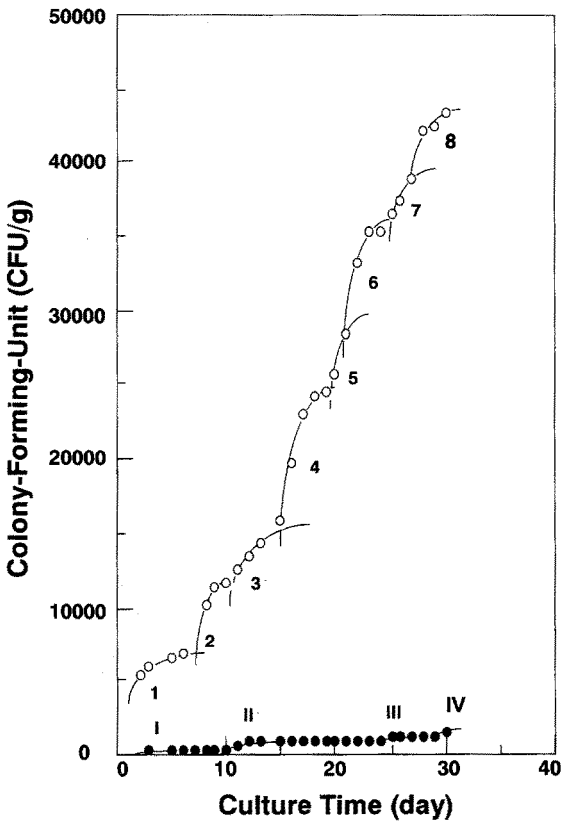

Fig. 2. Colony and clear-zone formation curves (CCFCs) of landfill leachate sample microorganisms on the YE-plate containing emulsified PHB. (○) Total colonies; (●) clear zones.

Table II. PHB-Degrading Microorganisms Isolated from Colonies Formed with Clear Zones on the YE- and NB-Plates

	Bacteria		Fungi	
	YE-plate	NB-plate	YE-plate	NB-plate
1. Landfill leachate (No. 1)	5	3		1
2. Landfill leachate (No. 2)	(1)[a]			1
3. Sewage sludge compost	4			
4. Sewage sludge supernatant	4	2	1	
5. Forest soil	2			
6. Farm soil	7	5	2	1
7. Paddy soil	9	4	2	
8. Weed field soil	3			
9. Roadside sand	4	2		
10. Pond sediment	7	6		
Total	45(1)	22	5	3

[a]Contaminants.

the consortium in the landfill leachate (No. 1) consisted of at least eight groups of microorganisms and at least four groups of PHB-degrading microorganisms differing in growth rates. CCFCs from other major samples also indicated that multiple groups of PHB degrading microorganisms were present.

A total of 75 strains was isolated from colonies formed with clear zones on the YE- and NB-plates (Table II). The majority of the strains was bacteria. It was confirmed that these strains depolymerized fibrous PHB in liquid culture.

Population and Distribution of Aerobic PHB- and PCL-Degrading Microorganisms

The colonies and clear zones on the agar YE-plates containing yeast extract (250 mg/liter) and emulsified PHB were counted for 30 days. The total number of microorganisms was in the range of 2.6×10^4 to 2.9×10^8 per g of sample, 9.9×10^5 to 7.0×10^9 per g of dry sample, and 7.6×10^6 to 1.2×10^{10} per g of organic substance. In several reports [13–16], the total population of bacteria and fungi in natural environments was reported to range from 10^7 to 10^9 per g of dry soil. In other reports [18, 19], the population of viable bacteria in lakes and rivers in Japan was estimated by the plate count method to range from 3.3×10 to 1.4×10^6 per ml. Therefore, the emulsified PHB does not appear to influence the growth of microorganisms on the YE-plate.

The relationship between total colonies (total microorganisms) and clear zones (degrading microorganisms) is shown in Fig. 3. In all samples, several kinds

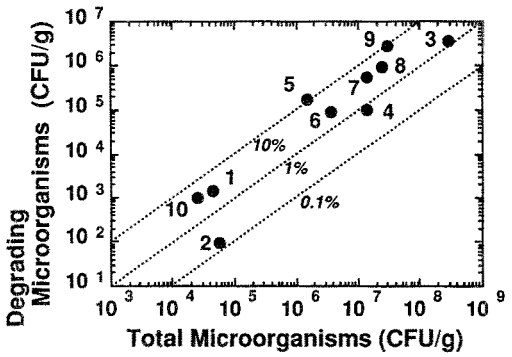

Fig. 3. Relationship between total microorganisms and PHB-degrading microorganisms formed on the YE-plates containing emulsified PHB; culture at 30°C for 30 days.

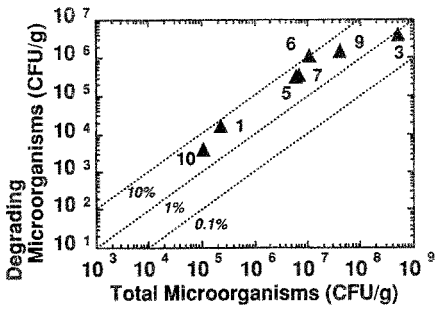

Fig. 5. Relationship between total microorganisms and PCL-degrading microorganisms formed on the YE-plates containing emulsified PCL; culture at 30°C for 10 days.

of colonies with clear zones formed. The percentage of PHB-degrading microorganisms to total colonies was 0.2–11.4%. It was clear that PHB-degrading microorganisms were distributed over all sample environments.

In the case of NB-plates containing nutrient broth (8000 mg/liter) and PHB, the colonies and clear zones were counted for 14 days. Thereafter, counting was no longer possible due to growth of fungi. The total number of microorganisms was in the range of 2.4×10^5 to 5.2×10^8 per g of sample, 2.2×10^6 to 7.9×10^9 per g of dry sample, and 1.1×10^7 to 1.3×10^{10} per g of organic substance. The growth rates of microorganisms on the NB-plates were usually rapid compared to the growth rates on YE-plates, because of the rich nutrients. The relationship between total microorganisms and PHB-degrading microorganisms is shown in Fig. 4. The percentage of degrading microorganisms to total microorganisms was 0.5–9.6%. These results are essentially the same as those obtained with YE-plates containing yeast extract (250 mg/liter).

In the case of PCL, the culture was made using seven soil samples for 10 days. The data for YE-plates containing yeast extract (250 mg/liter) and emulsified PCL are shown in Fig. 5. The total number of microorganisms was in the range of 1.1×10^5 to 5.0×10^8 per g of sample, 4.1×10^6 to 7.5×10^8 per g of dry sample, and 3.0×10^7 to 1.9×10^9 per g of organic substance. PCL also did not depress the colony formation of aerobic microorganisms on the agar plate. The percentage of PCL-degrading microorganisms to total colonies was 0.8–11.0%. PCL-degrading microorganisms were distributed over all sample environments.

The above results suggest that PHB- and PCL-degrading aerobic microorganisms are widely distributed as resident populations in many environments. In other words, PHB and natural PCL analogues may be distributed universally in the general environment. For example, cutin and suberin seem to be regarded as natural PCL analogues [22–27]. Brown and Kolattukudy reported that cutin was hydrolyzed by a lipase [28, 29]. It was, moreover, confirmed that several plant pathogenic microorganisms, which released cutinase [30–33], could form clear zones around the colonies on agar plates containing emulsified PCL. The details will be reported in future.

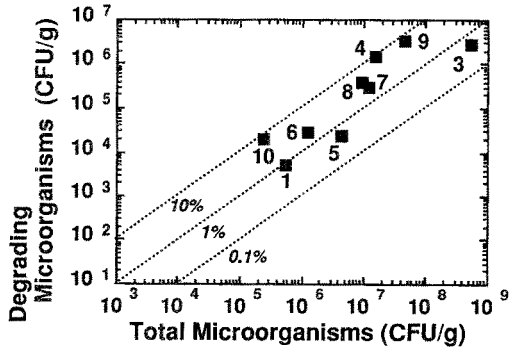

Fig. 4. Relationship between total microorganisms and PHB-degrading microorganisms formed on the NB-plates containing emulsified PHB; culture at 30°C for 14 days.

Relationship Between the Consortium of PHB-Degrading Microorganisms and PHB Degradation

The results in Figs. 3–5 indicate that populations of PHB-and PCL-degrading microorganisms are proportional to total microorganism populations. If the consortia of degrading microorganisms in the different samples are similar to each other, the capacity of the natural environment to degrade these plastics may be proportional to the population of degrading microorganisms or the percentage of degrading microorganisms to total microorganisms. Figures 6 and 7 show the relationships of PHB degradation by second enrichment population to

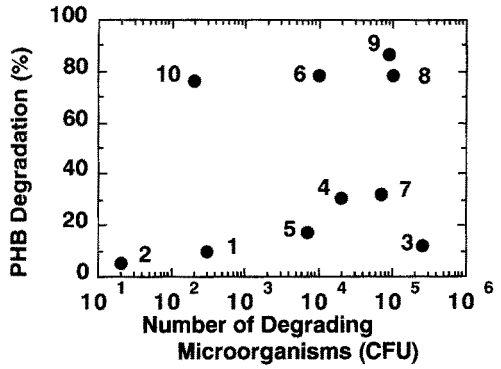

Fig. 6. Relationship between PHB degradation by the second enrichment population and the population of PHB-degrading microorganisms. Cultured at 30°C for 93.5 h (samples 1 and 2) or 45 h (samples 3–10).

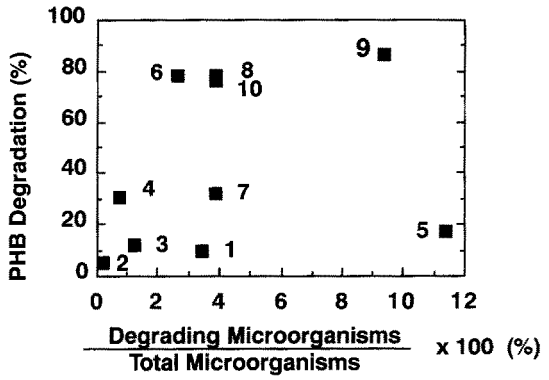

Fig. 7. Relationship between PHB degradation by the second enrichment population and the percentage of PHB-degrading microorganisms to the total microorganisms. Cultured at 30°C for 93.5 h (samples 1 and 2) or 45 h (samples 3–10).

the population and percentage of PHB-degrading microorganisms. No dependence could be found. It is thought that the degradation by enrichment population is influenced by several factors, such as the degradation abilities and growth rates of individual degrading microorganisms and the rapid growth of nondegrading microorganisms. The above results suggest that the consortia of degrading microorganisms in the different samples are not similar to each other.

To investigate the similarity of consortia of the PHB-degrading microorganisms in the soil samples, 28 strains isolated from colonies forming clear zones on YE-plates were investigated as to degradation behavior. The relationship between PHB degradation and clear-zone formation rate of the 28 isolated strains is shown in Fig. 8. The numbers marked in Fig. 8 represent the number of soil samples as isolation sources. No definite dependence could be detected. Thus, the majority of

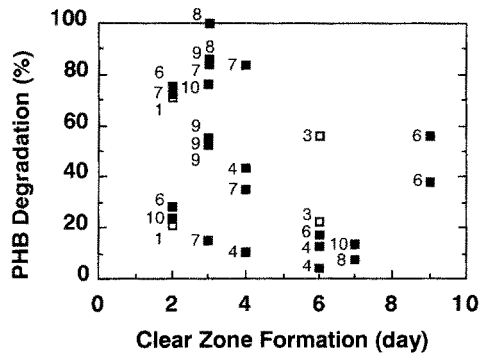

Fig. 8. Relationship between PHB degradation and clear-zone formation rate of the 28 strains isolated from the soil samples (Nos. 1–10). Cultured at 30°C for 46 h. The numbers marked represent the number of soil samples as isolation sources.

these strains would appear to differ from each other. These results indicate that the consortia of degrading microorganisms in the soil samples may be low in similarity. The taxonomic identification of the strains is in progress.

The high degradation activity of samples 6, 8, 9, and 10 in Figs. 6 and 7 may be due to the presence of microorganisms with a rapid growth rate and high degradation ability, as shown in Fig. 8. The low degradation activity of sample 3 may be due to the absence of active polymer-degrading microorganisms. However, different kinds of degrading microorganisms seem to show individual degradation activities under particular conditions.

If culture conditions, such as nutrients, pH, and temperature, are varied, the above results may also change remarkably. However, the above results show the significant suggestion that many kinds of PHB-degrading microorganisms are present in the natural environment and specific consortia differing in degradation capacity are probably present in each sample environment.

CONCLUSION

To assess the capacity of the natural environment for degrading (depolymerizing) plastic, agar plates containing emulsified PHB or PCL were used to count PHB- and PCL-degrading microorganisms in several environments, and the percentages of the degrading microorganisms to the total number of microorganisms were determined. PHB- and PCL-degrading microorganisms were shown to be distributed over all sample environments. The presence of many unidentified PHB- and PCL-degrading microorganisms was suggested. The

percentages of PHB- and PCL-degrading microorganisms were in the range of about 0.1–10%. In the general environment, PHB- and PCL-degrading microorganisms are present universally and may play an important role as resident microorganisms.

The relationship between the capacity of the natural environment for degrading PHB and the population or percentage of the PHB-degrading microorganisms could not be clarified. In each environment, a specific consortium differing in degradation capacity may be present. Classification of many unidentified degrading microorganisms and clarification of the relationship between the consortium and the degradation ability should be attempted in the future.

In the above manner, it is possible to assess the aerobic depolymerization of any polymer which is emulsified into an agar matrix. This estimation method is adaptable for anaerobic biodegradation of many other polymers. The results will be reported in the near-future.

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