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Confirmation of Anaerobic Poly(2-oxepanone) Degrading Microorganisms in Environments

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Anaerobic and ecological biodegradability of poly(2-oxepanone):poly(ε-caprolactone)(PCL) in different environments was estimated by the clear-zone method with anaerobic roll tube containing emulsified PCL. It was ascertained that PCL is degraded by anaerobic microorganisms and that anaerobic PCL-degrading microorganisms distribute considerably over natural environments.

The study of the biodegradation of plastics is important for solving the plastic waste problem. Many applications will include the use of the biodegradable plastics. The main materials that have been extensively researched with respect to biodegradation are aliphatic polyesters such as poly(β-hydroxybutyrate):(PHB) and poly(2-oxepanone):poly(ε-caprolactone)(PCL). PHB is a natural aliphatic polyester that is accumulated by many species of bacteria as storage material,[1] while PCL is a synthetic polyester. PCL is degraded rapidly and utilized by a number of aerobic microorganisms [2] and enzymes such as lipases [3] in spite of being a high molecular weight polymer. The anaerobic biodegradation of some other polymers, such as PHB, poly(ethyleneoxide), and poly(carboxylic acid), has been investigated,[4] but with regard to PCL no report has been published.

We have been investigating with respect to the distribution of polymer degrading microorganisms in natural environments with polymer emulsified agar plates. We reported that PHB and PCL-degrading aerobic microorganisms are widely distributed over many environments [5] and that poly(1,3-dioxolan-2-one)-degrading microorganisms are distributed in limited environments.[6]

In this report, we investigate with respect to the distribution of anaerobic PCL-degrading microorganisms in order to ascertain the anaerobic biodegradability of PCL and make sure that the estimation method with the polymer emulsified agar medium is adaptable for anaerobic biodegradation of polymers.

PCL (TONE P-767; number-average weight, 40000) was obtained from Union Carbide Corporation. Testing environmental samples from 10 locations were collected on 10 May 1992 from different environments at Tsukuba in Ibaraki Prefecture and environs: landfill leachate (No. 1 from Tokyo Bay), river water (No. 2 from the Sakura River), 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), creek sediment (No.8), roadside sand (No. 9), and pond sediment (No. 10).

Strict anaerobic techniques used for medium preparation were essentially the same as those of Hungate [7] as modified by Azuma and Suto to develop the gas jet method.[8] Traces of O_2 in the gases were removed

by passing the gases through a reduced copper column. Two roll-tubes containing PCL, one poor in nutrients (yeast extract, 250 ppm: YE-roll tube) and one rich in nutrients (GAM agar,[9] 74000 ppm: GAM-roll tube), were prepared. PCL (0.5 g) was dissolved in 20ml methylene chloride. The solution was emulsified with a homogenizer into 500 ml of a basal medium composed of 250 ppm yeast extract, 1000 ppm $(NH_4)_2SO_4$, 200 ppm $MgSO_4 \cdot 7H_2O$, 100 ppm NaCl, 20 ppm $CaCl_2 \cdot 2H_2O$, 10 ppm $FeSO_4 \cdot 7H_2O$, 0.5 ppm $Na_2MoO_4 \cdot 2H_2O$, 0.5 ppm $Na_2WO_4 \cdot 2H_2O$, 0.5 ppm $MnSO_4$, and 100 ppm 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 mM KH_2PO_4/K_2HPO_4. Agar (2.2%, W/V), 250 ppm cysteine·HCl·H_2O, 250 ppm $Na_2S \cdot 9H_2O$, and 1 ppm resazurin were added to the emulsified medium and the pH was adjusted to 7.1. The agar was dissolved by heating, which simultaneously evaporated methylene chloride from the medium. The medium was dispensed in 10 ml aliquots into 32-ml pressure glass tubes with butyl rubber stoppers. The gas phase of the tubes containing the agar medium was replaced with N_2 and the tubes were then autoclaved at 120 °C for 20 min. The tubes were held at 47 °C in a hot dry bath until inoculation with the testing environmental samples. The samples were diluted within the range of 10^{-1} to 10^{-4} with a dilution medium composed of the basal medium, 250 ppm cysteine·HCl·H_2O, 250 ppm $Na_2S \cdot 9H_2O$, and 1 ppm resazurin (pH 7.0), using 1-ml gassed sterile plastic syringes. The samples and diluted samples were inoculated into the agar medium containing the emulsified PCL, and then the roll tubes were formed (YE-roll tube).

In a similar manner as above, PCL was emulsified into GAM agar medium (pH 7.0) and roll tubes were again formed (GAM-roll tube).

The cultures were incubated stationary at 30 °C for 7 days. After the cultivation for 7 days, colonies and circular clear-zones formed in the PCL emulsified agar layer (Fig. 1) in the same manner as the cultivation of aerobic microorganisms on PCL emulsified agar plates.[5] The formation of clear-zones indicates that the colony cells at the center of a clear-zone degrade the surrounding emulsified polymer.[5,10]

The numbers of visible colonies and clear-zones in the roll tubes were counted. The relationships between both numbers of the total colonies (total culturable population) and the clear-zones (population of PCL-degrading microorganisms) are shown in Figs. 2 and 3. The populations of colonies and clear-zones were expressed as colony- or clear-zone-forming units (CFU) per 1 g of the testing enviromental sample.

The total culturable populations in the YE-roll tubes and GAM-roll tubes were in the range of 1.90 x 10^4 to 3.92 x 10^8 CFU g^{-1} and 1.11 x 10^4 to 1.25 x 10^6 CFU g^{-1}, respectively. The populations in the GAM-roll tubes were lower than those in the YE-roll tubes. It is considered that the GAM medium was too rich in nutrients for the testing environmental microorganisms to grow.[11]

The clear-zones were observed in 12 roll tubes which inoculated with 7 kinds of the testing natural environmental samples. These results indicate that anaerobic PCL-degrading microorganisms distribute over 7 sample environments; river water, sewage sludge supernatant, farm soil, paddy soil, creek sediment, roadside sand, and pond sediment. Usually, the total culturable populations in the roll tubes in which the clear-zones formed were lower than those in other roll tubes. It is assumed that the accumulation of hydrolysates and metabolites of PCL and the resultant pH drop [12] of agar medium suppressed the growth of other non-degrading microorganisms.

The populations of PCL-degrading microoganisms were in the range of 1.17 x 10^2 to 5.39 x 10^4 CFU g^{-1} (YE-roll tubes) and 7.04 x 10^2 to 4.00 x 10^4 CFU g^{-1} (GAM-roll tubes) and their percentages to the total culturable population were 0.6 - 4.5% and 0.9 - 12.9%, respectively. The percentages are equivalent to that of

aerobic PCL-degrading microorganisms (0.8 - 11.0%).[5]

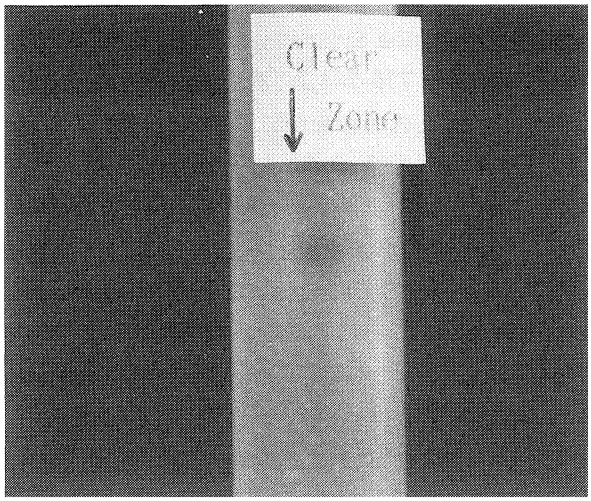

Fig.1. Colonies and a clear-zone in a YE-roll tube containing emulsified PCL.

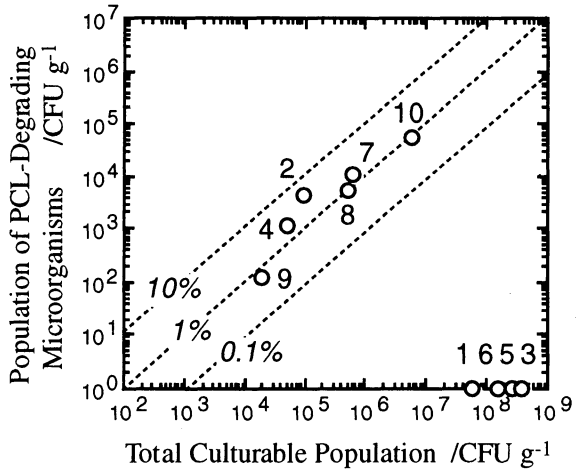

Fig.2. Relationship between total culturable population and PCL-degrading microorganisms formed in the YE-roll tubes containing emulsified PCL and yeast extract (250 ppm); culture at 30 °C for 7 days. The numbers marked represent the number of testing environmental samples.

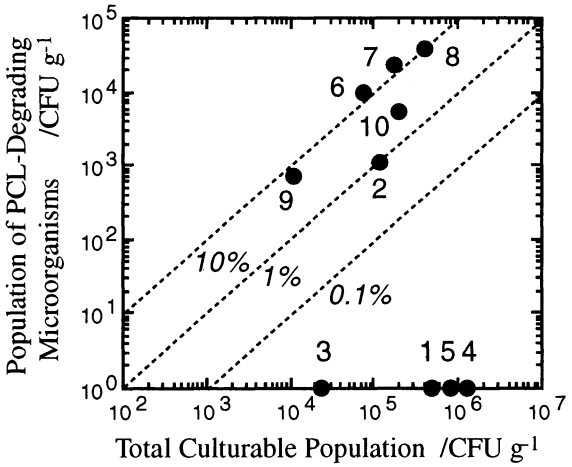

Fig.3. Relationship between total culturable population and PCL-degrading microorganisms formed in the GAM-roll tubes containing emulsified PCL and GAM medium (74000 ppm); culture at 30 °C for 7 days. The numbers marked represent the number of testing environmental samples.

The above results show that PCL is degraded by the anaerobic microorganisms and that the anaerobic PCL-degrading microorganisms distribute considerably over natural environments. A question arises as to why PCL is degraded by many microorganisms in natural environments. It is assumed that PCL is an analogue of some natural products which distribute over many environments.[5]

It was, moreover, ascertained that the estimation method with the polymer emulsified agar medium is adaptable for anaerobic biodegradability of polymers.

References

1) H. Brandl, R. A. Gross, R. W. Lenz and R. C. Fuller, *Advances in Biochemical Engineering/*

Biotechnology, **41**, 77 (1990).

2) R. D. Fields, F. Rodriguez, and R. K. Finn, *J. Appl. Polym. Sci.*, **18**, 3571 (1974); R. D. Fields and F. Rodriguez, Proceedings of the 3rd International Biodegradation Symposium, ed by J. M. Sharpley and A. M. Kaplan, Applied Science Publishers, London (1976), pp. 775-784; Y. Tokiwa, T. Ando, and T. Suzuki, *J. Ferment. Technol.*, **54**, 603 (1976); W. J. Cook, J. A. Cameron, J. P. Bell, and S. J. Huang, *J. Polym. Sci., Polym. Lett. Ed.*, **19**, 159 (1981); C. V. Benedict, W. J. Cook, P. Jarrett, J. A. Cameron, S. J. Huang, and J. P. Bell, *J. Appl. Polym. Sci.*, **28**, 327 (1983); C. V. Benedict, J. A. Cameron, and S. J. Huang, *ibid.*, **28**, 335 (1983).
3) Y. Tokiwa and T. Suzuki, *Agric. Biol. Chem.*, **41**, 265 (1977); Y. Tokiwa and T. Suzuki, *Nature*, **270**, 76 (1977); Y. Tokiwa and T. Suzuki, *Agric. Biol. Chem..*, **42**, 1071 (1978); Y. Tokiwa, T. Suzuki, and K. Takeda, *ibid.*, **50**, 1323 (1986); Y. Tokiwa, T. Suzuki, and K. Takeda, *ibid.*, **52**, 1937 (1988); K. Mukai, Y. Doi, Y. Sema, and K. Tomita, *Polym. Prepr., Jpn.*, **42**, 870 (1993).
4) P. H. Janssen and C. G. Harfoot, *Arch. Microbiol.*, **154**, 253 (1990); S. Matsumura and H. Shimokobe, *Chem. Lett.*, **1992**, 1859; B. Schink and M. Stieb, *Appl. Environ. Microbiol.*, **45**, 1905 (1983).
5) H. Nishida and Y. Tokiwa, *J. Environ. Polym. Degrade.*, **1**, 227 (1993).
6) H. Nishida and Y. Tokiwa, *Chem, Lett.*, **1994**, 421.
7) R. E. Hungate, "Methods in Microbiology," ed by J. R. Norris and D. W. Ribbons, Academic Press, Inc., New York (1969), Vol.3B, pp.117-132.
8) R. Azuma and T. Suto, Proceedings of the First International Conference on Culture Collections, ed by H. Iizuka and T. Hasegawa, University of Tokyo Press, Tokyo (1970), pp.493-505.
9) GAM agar medium (Nissui, medium No. 83 in Japan Collection of Microorganisms) was composed of the following (per liter): peptone 10.0 g, soybean peptone 3.0 g, proteose peptone w 10.0 g, serum dijest powder 13.5 g, yeast extract 5.0 g, beef extract powder 2.2 g, liver extract powder 1.2 g, glucose 3.0 g, KH_2PO_4 2.5 g, NaCl 3.0 g, soluble starch 5.0 g, cysteine HCl H_2O 0.3 g, Na_2S $9H_2O$ 0.3 g, and agar 15.0 g; N. Kosakai and S. Suzuki, "Anaerobes in Clinical Medicine," Igaku Shoin Ltd., Tokyo (1968), p.338.
10) J.Augusta, R.-J. Muller, and H.Widdecke, *Appl. Microbiol. Biotechnol.*, **39**, 673 (1993).
11) T. Hattori, *Rep. Inst. Agr. Res. Tohoku Univ.*, **27**, 23 (1976); H. Ohta and T. Hattori, *Soil Sci. Plant Nutr.*, **26**, 99 (1980).
12) H. Nishida and Y. Tokiwa, *Kobunshi Ronbunshu*, **50**, 739 (1993).

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