Journal of Environmental Polymer Degradation, Vol. 1, No. 1, 1993

Effects of Higher-Order Structure of Poly(3-hydroxybutyrate) on Its Biodegradation. II. Effects of Crystal Structure on Microbial Degradation

Haruo Nishida[1] and Yutaka Tokiwa[2]

As one of a series of studies concerning the relationship between the higher-order structure and the biodegradability of a biodegradable plastic, the effects of the crystal structure of the plastic on microbial degradation were investigated. Bacterial poly(D-(−)-3-hydroxybutyrate) (PHB) films which had a wide range of crystallinity were prepared by the melt-quenching method. Results of the microbial degradation indicated that the development of crystallinity evidently depressed the microbial degradability. From scanning electron microscopy (SEM) observations, it is suggested that the microbial degradation proceeded in at least two manners. One was preferential degradation of the amorphous region leaving the crystalline lamellae intact, which was considered to be a homogeneous enzymatic degradation over the surface. The other was nonpreferential spherical degradation on the surface. The SEMs indicate that the spherical holes were the result of colonization by degrading bacteria. The holes varied in size and number with the change of crystal structure. Therefore, it is considered that the crystal structure of PHB also influenced the physiological behavior of the degrading bacteria on the PHB surface.

KEY WORDS: Biodegradable plastic; microbial degradation; bacterial poly(D-(−)-3-hydroxybutyrate); crystal structure; physiological behavior.

INTRODUCTION

The study of the biodegradation of plastics is an important approach to solving the plastic waste problem.

Generally, plastics are water-insoluble materials and have higher-order structures that are too complex to envisage from each simple monomeric unit. The characteristic properties of plastics are manifested and controlled by the higher-order structures. Although many reports relating to the microbial and enzymatic degradation of plastics have been published, these have mainly dealt with the relationship between degradability and first-order structures such as the chemical structure and composition. The effects of the higher-order structure of plastics on biodegradation need to be clarified. These will lead to a concrete method for solving the problem of plastic waste.

Main materials that have been extensively researched with respect to biodegradation are polycaprolactone (PCL) and bacterial poly(D-(−)-3-hydroxybutyrate) (PHB). PCL is a synthetic high molecular weight polyester. Huang and Cameron and colleagues [1–3] investigated the effect of molecular weight, crystallinity, and morphology on the fungal degradation of crystalline PCL qualitatively and suggested that the dominant factor determining degradability was either molecular weight or crystallinity and that the fungal degradation proceeded in a selective manner, with amorphous regions being degraded prior to the degradation of crystalline regions. However, they also found that the degradation of PCL by *Penicillium funiculosum*

[1] Tsukuba Research Laboratory, Tokuyama Soda Co. Ltd., 40 Wadai Tsukuba, Ibaraki 300-42, Japan.
[2] Fermentation Research Institute, Agency of Industrial Science and Technology, 1-1-3 Higashi Tsukuba, Ibaraki 305, Japan.

1064-7564/93/0100-0065$06.50/0

proceeded in the crystalline regions as well as the amorphous regions. These results indicate that the relationship between overall molecular organization of plastics and physiological behavior of degrading microorganisms needs to be clarified more correlatively.

PHB is a thermoplastic in spite of being a macromolecular substance of natural origin. Further, PHB has a simple monomeric unit and a complex higher-order structure. This material may be regarded as an interface between a natural macromolecular substance and a synthetic polymer. Various information relating to the higher-order structure [4–11] and the biodegradation [12–21] of PHB is available, but there are few reports relating to the relationship between the higher-order structure and biodegradation of PHB. Merrick *et al.* [22] reported that native PHB granules possess definite structural features, disruption of which results in decreased susceptibility of the polymer to enzymatic hydrolysis. Kawaguchi and Doi [23] reported that crystallization of native granules was caused by the removal of lipid components by various treatments. Holland *et al.* [24] examined the effect of specimen preparation technique on hydrolytic degradation and showed that various forms of PHB and copolyesters had differing stabilities to hydrolytic attack. Kunioka *et al.* [25] and Doi *et al.* [26] investigated the crystalline structures and enzymatic degradation of PHB and copolyesters, and reported that the chemical composition of copolyesters affected the enzymatic degradability more than the crystallinity.

Recently, we investigated the microbial degradation of heat-treated PHB cast films [27]. It was found that the higher-order structure properties, such as crystallinity, modulus of elasticity, etc., acted as suppression factors. From SEM observations of the degraded film surface, unique degradation patterns, such as spherical holes, were found, and on the inside surface of the holes many cells of degrading bacteria in the process of degrading the PHB surface were observed. This degradation pattern was thought to result from colonization by the degrading bacteria. These results suggest that the surface of PHB is an important microenvironment for the growth of degrading bacteria and that the ecological and physiological behavior of the degrading bacteria in this environment was an essential factor in microbial degradation of plastic.

This report describes the investigation of the effects of crystal structure on microbial degradation of PHB, as one important factor of the higher-order structure. PHB samples having a wide range of crystallinity were prepared by the melt-quenching method. It was found that the development of crystallinity evidently depressed the microbial degradability. SEM observations suggest that the microbial degradation proceeded in at least two manners, one being homogeneous enzymatic degradation over the sample surface and the other spherical degradation resulting from localized torrential enzymatic action with colonization of degrading bacteria. The influence of the crystal structure of PHB on physiological behavior of degrading bacteria is 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 22×10^4 from intrinsic viscosity data according to Einaga's method [28].

Melt-Quenched Samples

PHB films were prepared by a conventional solvent casting technique from solutions of the reprecipitated fibrous PHB in chloroform. Cast films (initial film dimensions: 16 mm × 12 mm × 500 μm; initial weight: 80–100 mg) were set in molds, melted at 190°C in an oven, and rapidly quenched by soaking in a liquid such as hexane or liquid paraffin maintained in a thermostat. Quenching temperatures were set at four or five levels (−80°C, 0°C, 60°C, 130°C or −80°C, 0°C, 30°C, 80°C, 130°C). Quenched films were allowed to crystallize isothermally at each quenching temperature for 10 h. Immediately prior to the microbial degradation test, the samples were washed in hexane and dried *in vacuo* at room temperature for 2 h and the X-ray diffraction patterns thereof were measured at room temperature. The microbial degradation tests were run immediately after the X-ray crystallinity measurements.

Wide-angle X-ray diffraction diagrams of the quenched samples were recorded by the flat film method using Cu $K\alpha$ radiation ($\lambda = 0.154056$ nm) with a Rigaku Rotaflex system operated at 50 kV and 24 mA.

Wide-angle X-ray diffraction profiles were recorded in the range of $2\theta = 6$–$40°$ at a scan speed of 2°/min using Cu $K\alpha$ radiation with a Rigaku Rint-1200 system. The percentage of crystallinity was calculated from diffracted intensity data according to Tsunoda's method [29].

Polarizing microscopy observations were carried on

the quenched films (50–500 μm thick) using an Olympus SZH system and a Nikon Optiphot-POL system.

PHB-Degrading Bacterium Strain SC-17

Farm soil, obtained from a taro field at Tsukuba, Japan, was incubated in a medium composed of 0.25% purified fibrous PHB, 10 ppm $FeSO_4 \cdot 7H_2O$, 200 ppm $MgSO_4 \cdot 7H_2O$, 1000 ppm $(NH_4)_2SO_4$, 20 ppm $CaCl_2 \cdot 2H_2O$, 100 ppm NaCl, 0.5 ppm $Na_2MoO_4 \cdot 2H_2O$, 0.5 ppm Na_2WO_4, 0.5 ppm $MnSO_4$, and 100 ppm yeast extract in 10.7mM KH_2PO_4/K_2HOP_4 (pH 7.1) under vigorous aeration at 30°C for several days until the PHB was disintegrated and turbidity of the medium developed. A pure culture was obtained by repeated application of subcultures on the same media and plating the cultured bacteria on the above medium containing 1.5% agar. An isolated strain (SC-17) was maintained on an agar slant at 5–10°C after incubation at 30°C for 72 h.

Alcaligenes paradoxus [13, 15] (ATCC 17718) and *Pseudomonas testosteroni* [13, 15] (ATCC 17510) were also used for the PHB degradation test.

PHB Degradation Tests

A medium identical with the above culture medium except for the addition of the PHB was used for the degradation test. After the test medium contained in a 500-mL flask had been autoclaved at 120°C for 20 min, one series of samples melt-quenched at each temperature was added followed by the injection of the bacterium. The flask was aerated by stirring with a rotary shaker at 180 rpm and 30°C. After an incubation period, the remaining samples were taken out, vigorously washed by fresh water, and dried *in vacuo* (1–3 mm Hg) at room temperature for 1 night. Degradation values were calculated from the weight loss of the samples. The degraded samples were kept at −80°C for 6–10 days until the second X-ray crystallinity measurements were taken.

The microbial degradation tests were replicated 4 times.

Control experiments were run without the injection of the bacterium and replicated 2 times.

Scanning Electron Microscopy (SEM) Observations

After the microbial degradation tests, PHB samples were post-treated as mentioned above. In order to observe cross-sectional appearances, the degraded and control samples were broken in liquid nitrogen. Surfaces and cross-sectional appearances of the samples were observed using a JEOL model JSM-T220 with 15 kV acceleration after Au coating of the samples using an ion coater.

RESULTS AND DISCUSSION

Crystal Structure of Melt-Quenched Samples

PHB films melted at 190°C were quenched rapidly by soaking in a liquid such as hexane or liquid paraffin in a thermostat. Crystallization was carried out isothermally by aging at each quenching temperature for 10 h. Crystallization under more severe conditions, such as at 150°C for 40 h, led to liquefaction caused by thermal degradation as reported by Grassie *et al.* [30]. Any considerable decrease in molecular weight or change in surface condition has to be avoided, as these may influence the microbial degradation.

X-ray diffraction diagrams of the samples showed some Debye–Scherrer rings. This indicates that the samples do not have a crystals orientation. Figure 1 shows the X-ray diffraction profiles of the melt-quenched samples just before the microbial degradation test. X-ray crystallinity increased from 42 to 72% with the rise in the quenching temperature (Fig. 2). The range of crystallinity of the melt-quenched samples was larger than that of heat-treated samples, which changed from 56 to 64%, as reported previously [27].

The microbial degradation tests were run at 30°C for 84 h immediately after the initial X-ray crystallinity measurements. After the degradation tests, the degraded and control samples were dried *in vacuo* at room temperature for 1 night and kept at −80°C for 6–10 days until the second X-ray crystallinity measurements. Figure 2 shows the changes in the crystallinity values of control samples between the initial and second measurements. Taking into account the glass transition temperature (T_g, −5 ~ 5°C [8]), spherulite growth rate [8], and the nucleation rate [9] of PHB, it is considered that the changes of crystallinity values in Fig. 2 occurred predominantly during the microbial degradation tests. Every control sample except the 130°C-quenched sample (Sample 130) showed an increase in crystallinity. In particular, the values of samples quenched at 0 and −80°C (Samples 0 and −80) changed remarkably because the quenching temperatures were nearly equal or lower than the T_g of PHB. However, the order of crystallinity was unchanged.

In Fig. 3, the series of optical micrographs shows the change in crystalline morphology with the rise in quenching temperature. Sample −80 consisted of only

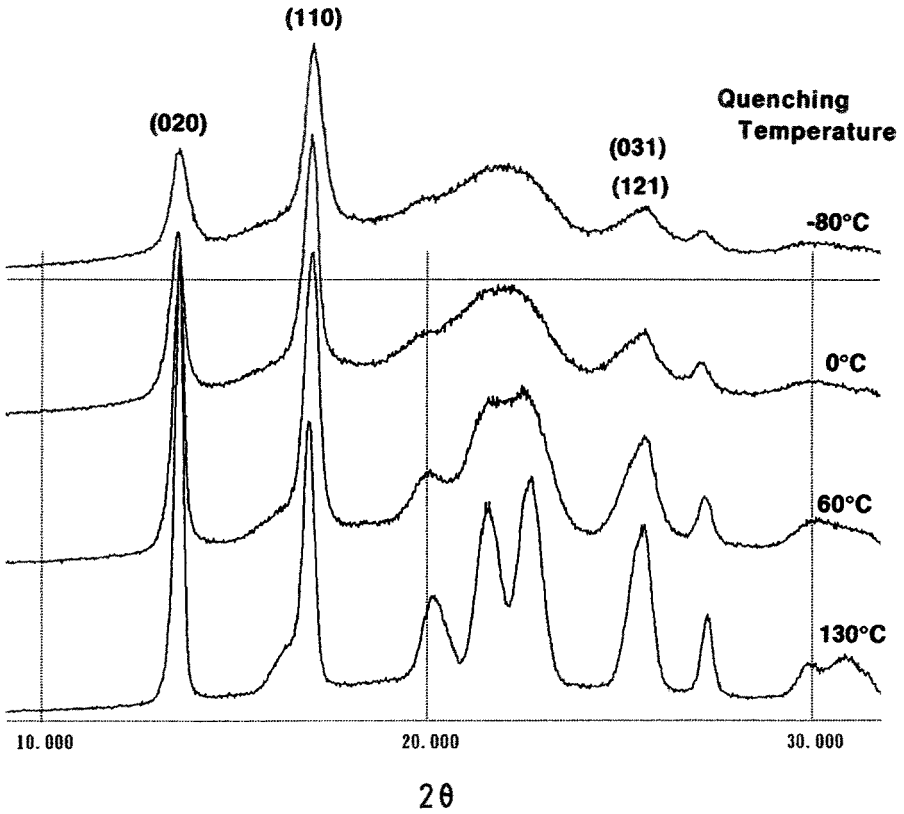


Fig. 1. X-ray diffraction profiles of melt-quenched PHB samples before microbial degradation test. Samples melted at 190°C were rapidly quenched by soaking in a liquid such as hexane or liquid paraffin at each quenching temperature and then maintained at the same temperatures for 10 h. After drying *in vacuo* at room temperature for 2 h, X-ray diffraction profiles of the samples were recorded.

crystallites. Sample 0 contained crystallites and some spherulites smaller than 50 μm. In Sample 60, the spherulite diameter is in the range of 100–200 μm and there is a Maltese cross pattern. Sample 130 has the largest spherulites (200–500 μm) but there are no Maltese cross patterns. Similar observations of PHB spherulites were reported by Barham and colleagues [8–10]. They indicated the variation of the average radius of PHB spherulites as a function of their crystallization temperature [8]. The report [10] also described large spherulites without a Maltese cross pattern.

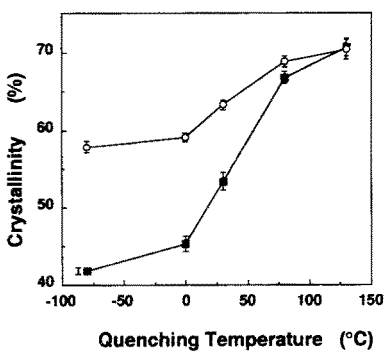


Fig. 2. Changes of crystallinity values of melt-quenched PHB control samples during the microbial degradation test. ■, Initial; ○, after the test at 30°C for 84 h; bar = SD.

As shown in the above results, the melt-quenched PHB samples exhibited several types of crystalline morphology with changes in quenching temperature. With changes in crystal structure, the surface morphology also varied (Fig. 4).

Microbial Degradation of Melt-Quenched Samples

Microbial Degradability

PHB samples quenched at five temperatures, namely, −80, 0, 30, 80, and 130°C, were used for the microbial degradation test. Microbial degradation was

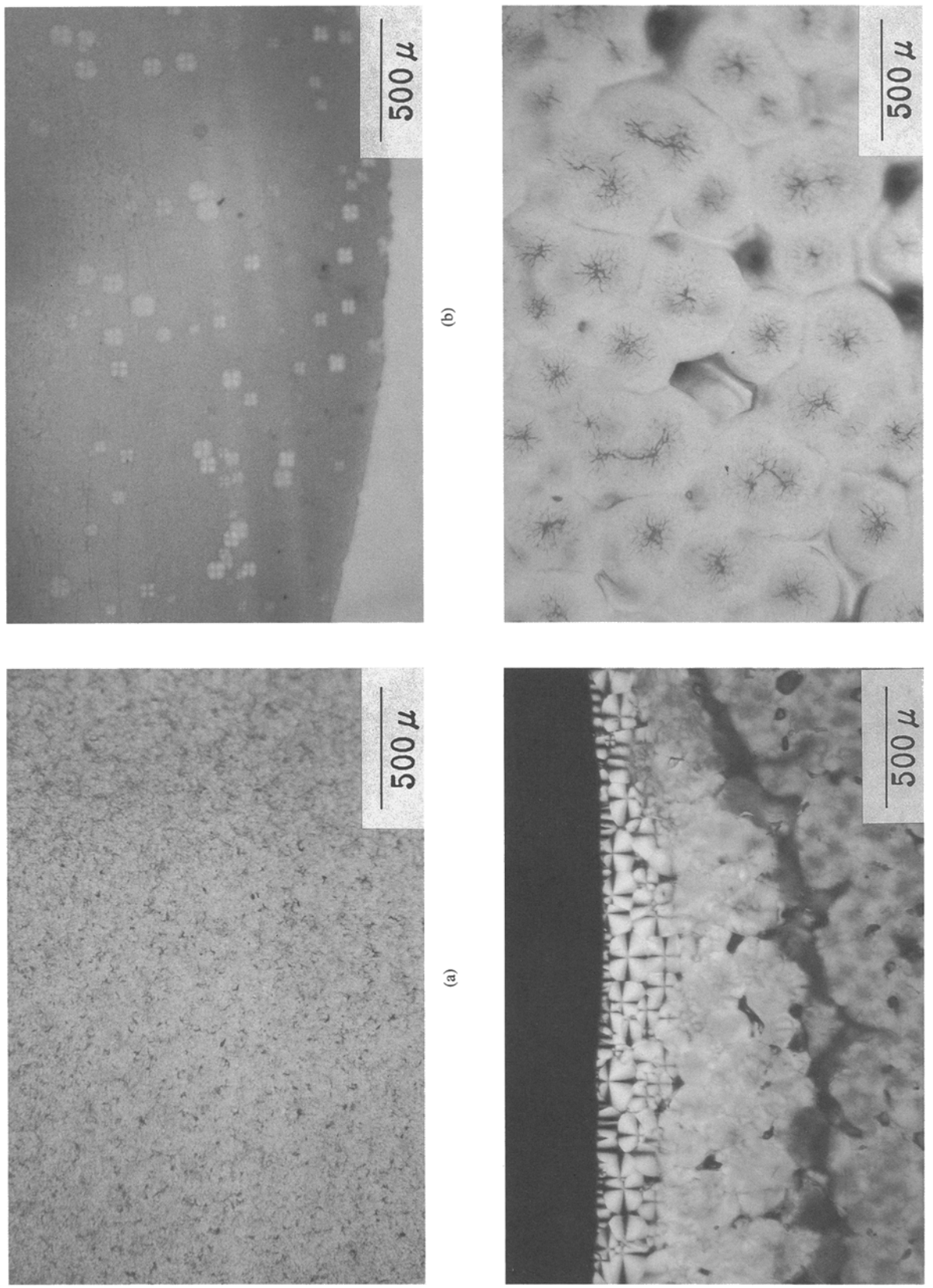


Fig. 3. A series of optical micrographs with crossed polarizers showing how the crystal morphology of PHB varies with quenching temperature (T_q). T_q = (a) −80; (b) 0; (c) 60; and (d) 130°C.

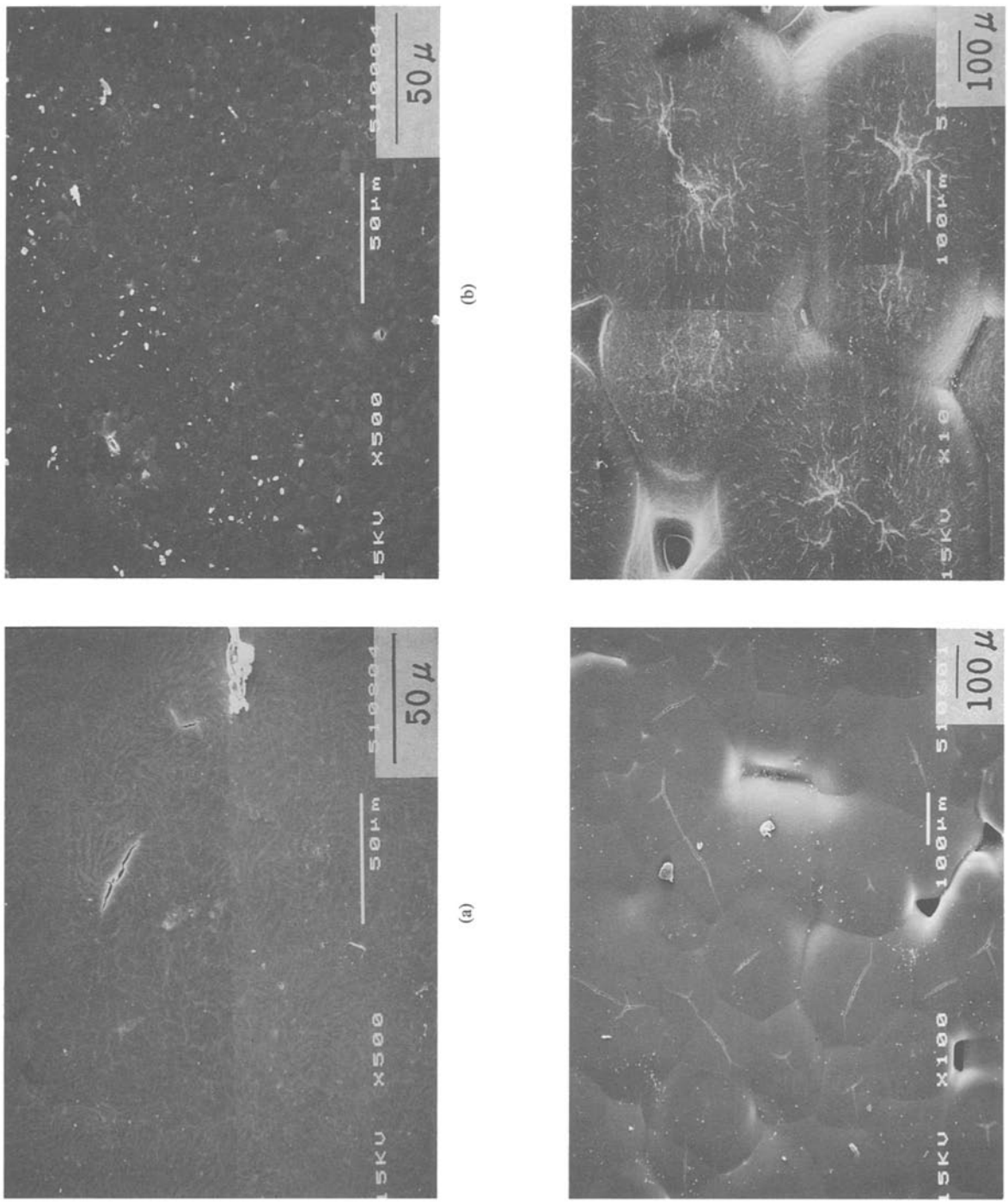


Fig. 4. SEMs of control PHB samples surfaces after cultivation at 30°C for 72 h without injection of bacterium. (a) Sample −80; (b) Sample 0; (c) Sample 60; (d) Sample 130.

carried out using three kinds of aerobic bacteria: strain SC-17, *Alcaligenes paradoxus* [13, 15] (ATCC 17718), and *Pseudomonas testosteroni* [13, 15] (ATCC 17510) at 30°C for 84 or 24 h. Strain SC-17, which was obtained from a taro field at Tsukuba, Japan, by an enrichment culture procedure, was chiefly used. The excretion of extracellular depolymerase by strain SC-17 was evidenced by clear zones surrounding the colonies of SC-17 on a mineral-agar plate containing emulsified PHB.

An important and effective factor in microbial degradation is the concentration of the degrading bacteria in the culture medium. If a substrate is degraded easily, the degrading bacterium also propagates rapidly. Consequently, the degradation is accelerated by the multiplied effects. This being the case, in the degradation test, a series of samples quenched at different temperatures was added to the same culture medium.

Figure 5 shows the microbial degradation of the melt-quenched samples as a function of quenching temperature. Degradation decreased with the rise in quenching temperature in every test using the three kinds of degrading bacteria. These results could be duplicated. The results in Fig. 5 indicate that the microbial degradation is depressed by the crystal of PHB. It also suggests that the amorphous region is degraded selectively or preferentially. Huang and Cameron and colleagues indicated that the crystallinity of polycaprolactone (PCL) was a dominant factor determining degradability on the fungal degradation [2] and that the fungal degradation of crystalline PCL proceeded in a selective manner, with the amorphous regions being degraded prior to the degradation of the crystalline regions [1, 3]. It is considered that the results in Fig. 5, where every sample had the same molecular weight, confirm their ideas.

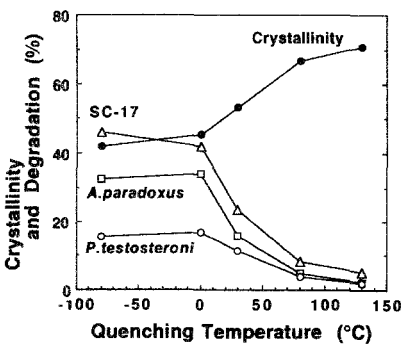


Fig. 5. Microbial degradation of melt-quenched PHB samples of different crystallinity by three kinds of degrading bacteria at 30°C. ●, Crystallinity (%); △, strain SC-17 (culture time = 84 h); □, *Alcaligenes paradoxus* (culture time = 24 h); ○, *Pseudomonas testosteroni* (culture time = 24 h).

Changes in Crystallinity Caused by Microbial Degradation

The X-ray crystallinity values of the quenched samples after microbial degradation by strain SC-17 were measured by the reflection method (Fig. 6). The measurement was replicated 4 times or more than for all samples in Fig. 6. Standard deviation (SD) values also are shown as error bars in Fig. 6. The results in Fig. 6 indicate that the crystallinity values of each of the degraded samples are slightly lower, or at least not higher, than those of the corresponding control samples. This is considered to be an indirect indication that selective or remarkable preferential degradation of the amorphous region does not take place. Doi *et al.* [31] reported that the crystallinity of a copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate increased with hydrolysis in a phosphate buffer at 70°C and pH 7.4. Therefore, the above results suggest that microbial degradation proceeds in a different manner from chemical hydrolysis.

SEM Observations of Degraded PHB Samples

To clarify the degradation behavior of the degrading bacteria, SEM observations of cross sections and surfaces of the degraded samples were carried out.

PHB samples quenched at four temperatures, namely, −80, 0, 60, and 130°C, were used for the microbial degradation by strain SC-17 and followed SEM observations. The degradation results at 30°C for

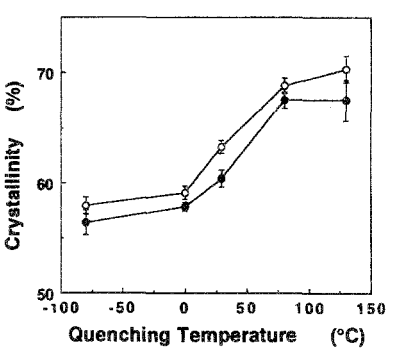


Fig. 6. Changes in crystallinity values of melt-quenched PHB samples after microbial degradation. ●, After cultivation of strain SC-17 at 30°C for 84 h; ○, control; bar = SD.

72 h were similar exactly in tendency to the results shown in Fig. 5.

Cross-Section Observations

Figures 7A and 7B are SEMs of cross sections of the degraded samples. In the SEMs of Samples −80, 0, and 60 (Fig. 7A), in which the weight loss values were 55.3, 48.1, and 17.0%, respectively, spherical holes were found on each surface. As reported previously [27], similar holes were found on the surface of degraded cast film, and were assumed to be caused by colonization by strain SC-17. In the SEMs of Samples 60 and 130 (Fig. 7B), the lamellae of the spherulites are left in relief. In all samples, degradation took place at the surface layer and the inside portions of samples apparently were unchanged. This surface erosion supports the above result that the crystallinity values of degraded samples were not higher than those of control samples. Doi *et al.* [26] reported that the enzymatic degradation of PHB cast film proceeded as surface erosion. But, a spherical degradation pattern such as the above has not been observed. It is considered that the spherical degradation pattern is due to the microbial action.

Surface Observations

SEMs of the degraded sample surfaces are shown in Fig. 8. Spherical holes of about 20 μm in diameter were formed over the whole surface of the degraded Samples −80 and 0 [Fig. 8(a) and (b)]. This degradation pattern was similar in shape, size, and number to that formed on the surface of degraded cast film [27]. This means that the surfaces of Samples −80 and 0 are similar in some conditions for the microbial degradation to the cast-film surface. As a reason for the similarity, it is thought that the crystals in these samples aren't so large as to be recognizable by the degrading bacterium as an unusual area. The same degradation pattern was also found in cases of degradation using other degrading bacteria, namely, *Alcaligenes paradoxus* and *Pseudomonas testosteroni*.

In the case of Sample 60, which contained spherulites 100–200 μm in diameter, the lamellae of spherulites were left in relief and there were also larger spherical holes here and there on the degraded surface [Fig. 8(c)]. The relief of lamellae indicates preferential degradation of the amorphous region between the lamellae at the surface layer, while the spherical holes signify nonpreferential degradation with no distinction between crystal and amorphous regions.

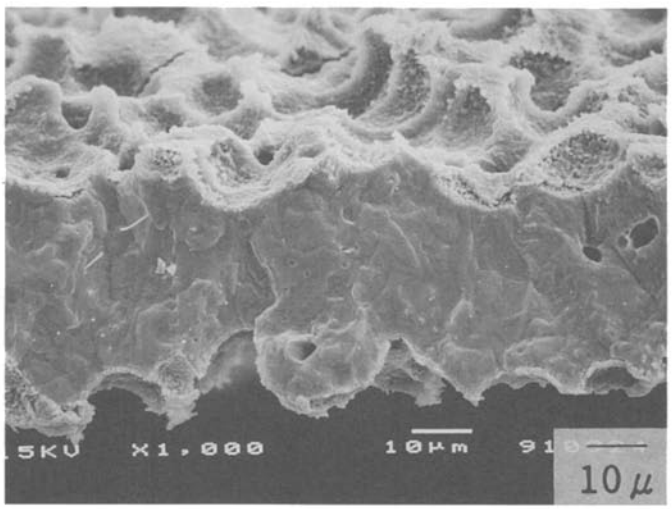
Cook *et al.* [1] reported that the degradation of PCL by *Penicillium funiculosum* proceeded in the crystalline regions as well as the amorphous regions and that the degradation along the mycelia was sufficient to cause the mycelia to sink into the surface of the film. It is considered that the spherical holes and the degradation along the mycelia are identical.

An enlarged view of the inside surface of a spherical hole showed whiskers of crystal lamellae remaining. This indicates that fundamentally the degradation of the inside surface of the spherical holes also proceeds in the preferential degradation manner of amorphous regions.

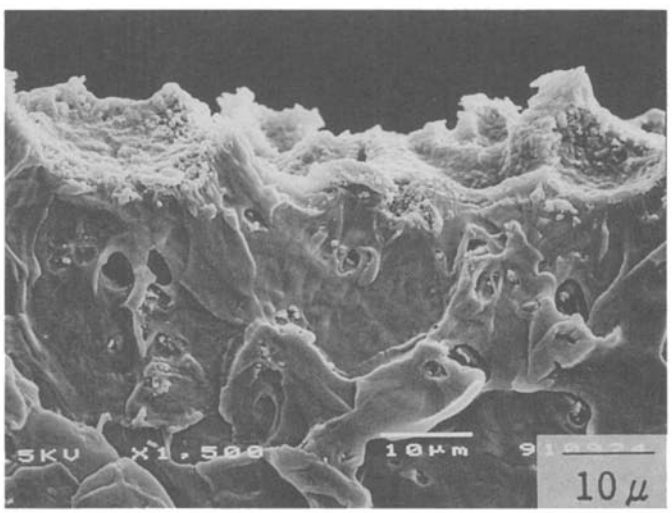
On the surface of the degraded Sample 130, in which the weight loss value was 3.4%, the preferential degradation of amorphous regions was recognized but no spherical holes were observed [Fig. 8(d)]. To examine the possibility of the spherical holes formation on the surface Sample 130, which consists of large spherulites, the time course of the surface degradation pattern of Sample 130 was studied. After a fixed incubation period at 30°C, a sample degraded by strain SC-17 was taken out, vigorously washed by fresh water, and dried *in vacuo* at room temperature. The SEM observations—results of follow-up until the weight loss reached 40.1%—are shown in Fig. 9. The preferential degradation of amorphous regions was observed, but there was little formation of spherical holes on the surfaces. Erosion of the ring structures of surface spherulites proceeded characteristically. Finally, the surface was evened out and the crystalline lamellae remained as whiskers on the surface.

The SEMs of Figs. 8 and 9 indicate that the degradation manner of strain SC-17 varies individually with the change of crystal structure of PHB. In particular, the spherical holes on the surface were significantly influenced in the size, number, and even the formation itself.

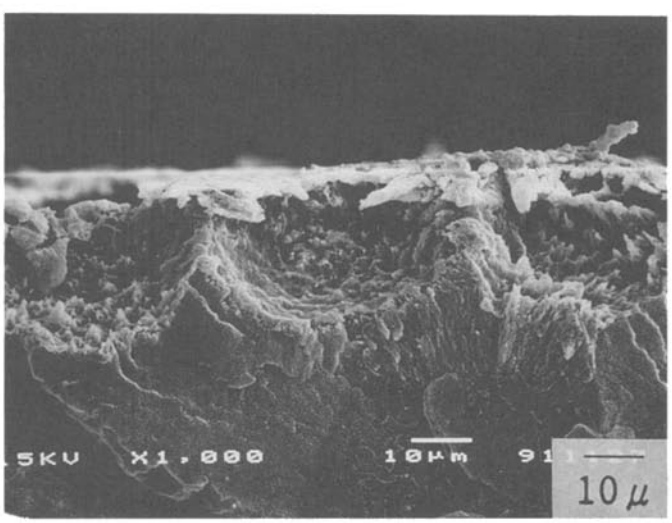
The above results suggest that the microbial degradation of the crystal structure of PHB proceeds in at least two manners. One is the preferential degradation of amorphous regions at the surface layer, leaving the crystalline lamellae in relief, and the other is nonpreferential degradation, forming spherical holes on the surface. It is presumed that the preferential degradation of amorphous regions is due to homogeneous enzymatic action over the surface by diffused extracellular depolymerases and other degradation results from localized torrential enzymatic action with colonization of degrading bacterium. Therefore, it is considered that the change of crystal structure of PHB affects the colonization or physiological action of the degrading bacterium on the PHB surface.



(a)



(b)



(c)

Fig. 7A. SEMs of cross section of degraded PHB samples showing the spherical holes on the surface. (a) Quenching temperature (T_q) = −80°C (Sample −80); (b) Sample 0; (c) Sample 60.

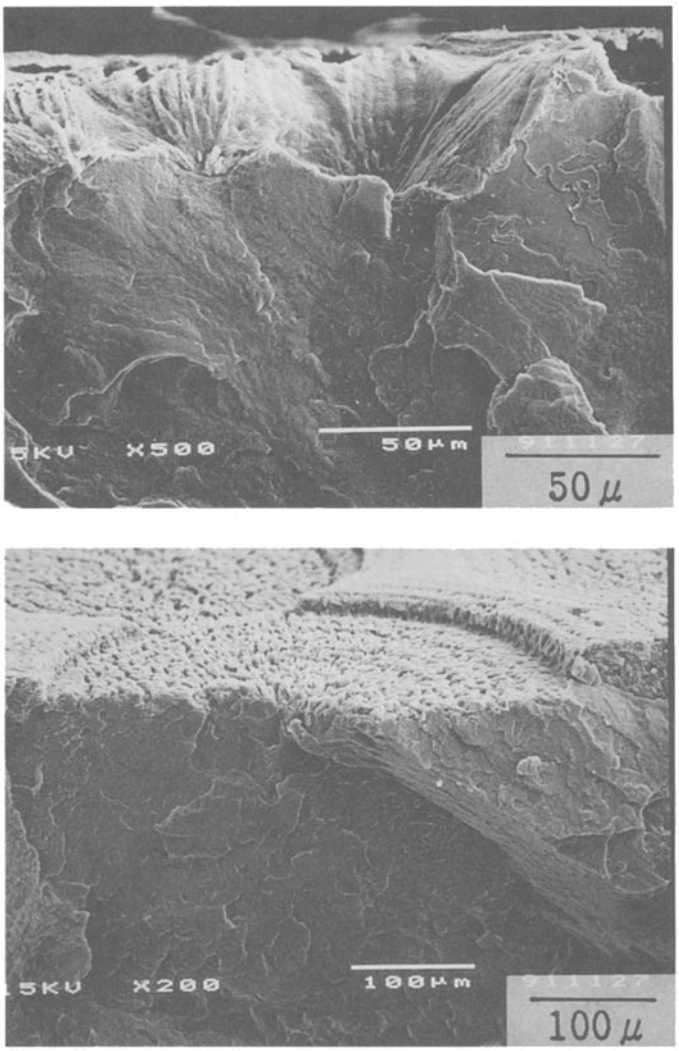


Fig. 7B. SEMs of cross section of degraded PHB samples showing the lamellae of spherulites. (c) Quenching temperature (T_q) = 60°C (Sample 60); (d) Sample 130.

In the following investigation, the physiological action of the bacterium on the surface was observed.

Physiological Action of Degrading Bacteria on the Surface

Figure 10 shows an enlarged view of the degraded crystalline lamellae on the surface of degraded Sample 60. In Fig. 10, the cells of strain SC-17 adhere to or get in among the lamellae. However, the intrusion of cells among the lamellae is not predominant. This SEM, therefore, suggests that the cells on lamellae adhere to the spherulite surface and degrade preferentially the amorphous region among the lamellae by extracellular depolymerases.

Figure 11 shows enlarged views of a spherical hole on the surface of the same sample. The hole appears to be filled with the cells of strain SC-17. The cells were straight rods and 1 × 2–3 μm in size. The morphology was about the same as we observed under phase contrast microscopy. The picture was similar in the case of a spherical hole formed on the cast-film surface after degradation by strain SC-17 [27]. It is, therefore, confirmed that the nonpreferential spherical degradation pattern on the surface results from colonization by the degrading bacterium SC-17 on the sample surface. Zobell [32] and

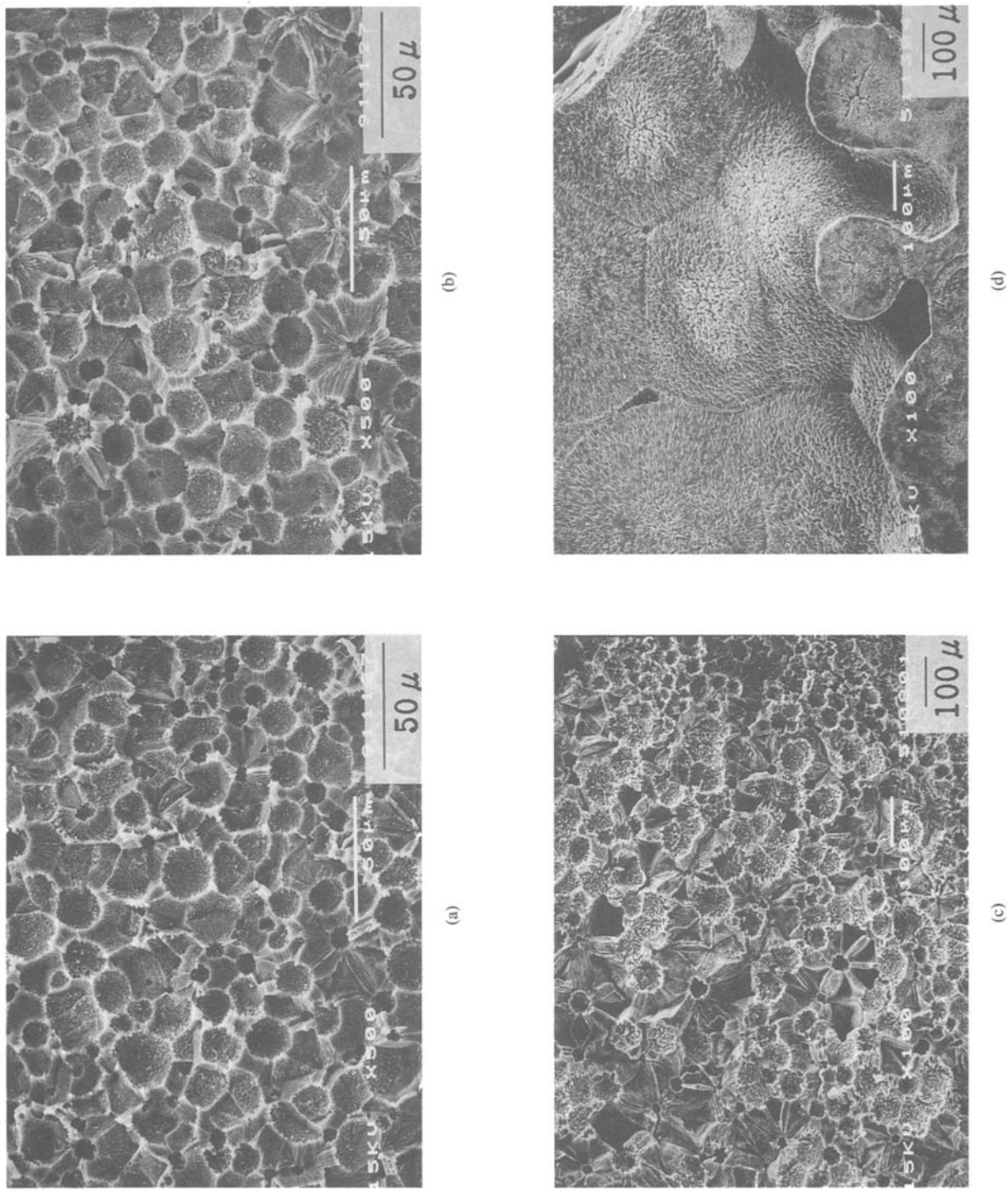


Fig. 8. SEMs of degraded PHB samples surfaces after cultivation at 30°C for 72 h by strain SC-17. (a) Sample −80; (b) Sample 0; (c) Sample 60; (d) Sample 130.

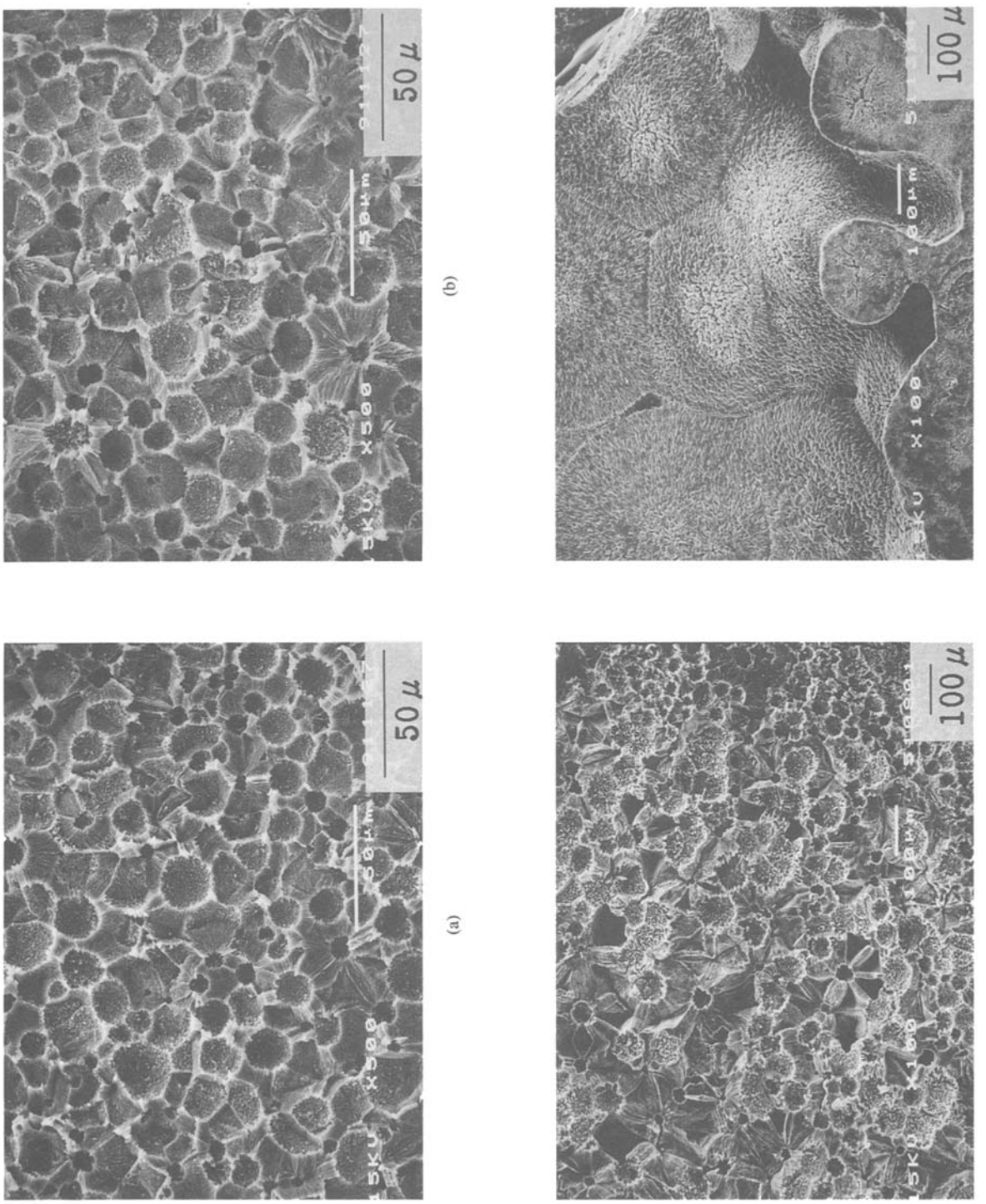


Fig. 9. Time-course change in surface morphology of Sample 130 with microbial degradation by strain SC-17. (a) Culture time = 0 h, weight loss = 0%; (b) 69 h, 6.2%; (c) 189 h, 24.6%; (d) 333 h, 40.1%.

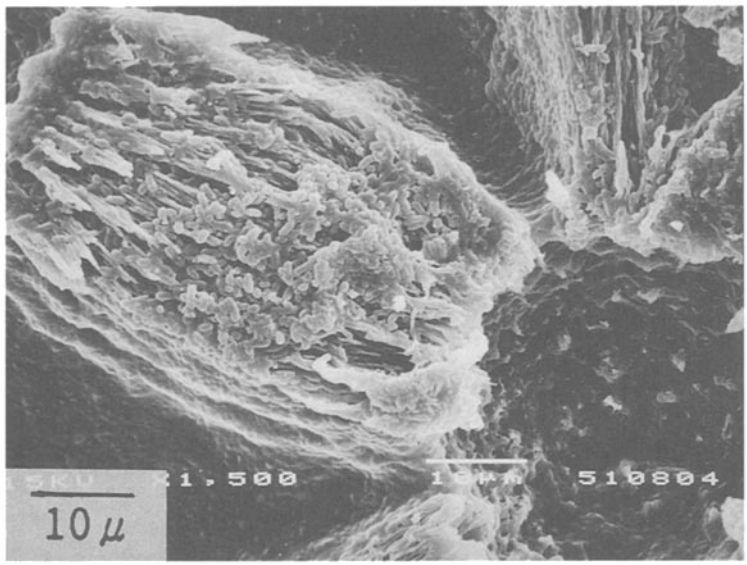


Fig. 10. SEM of surface of degraded spherulite of PHB Sample 60 after cultivation at 30°C for 72 h by strain SC-17.

Hattori *et al.* [33] suggested that the liquid–solid interface had a significant influence on bacterial growth or activities. The physiological action of the bacterium on the PHB film is more significant, because the PHB is a solid material and furthermore the sole substrate.

Colonization Point on the PHB Surface

As shown in Fig. 8, the size, number, and even the formation itself of spherical holes were significantly influenced by changes in the crystal structure. This fact suggests that the colonization is somewhat selective with respect to the starting point. Figure 8(a), (b), and (c) show spherical holes formed at various places, but it is noted that the holes tend to be formed at a crystal center and on a boundary line between spherulites. Figure 12(a) and (b) are the enlarged view of the surface of Samples −80 and 0, respectively, and show the formation of the spherical holes at the center of crystals and on the boundary lines. There appears to be a circular degradation pattern at the center of the spherulite. It is thought that a structural problem in the spherulite concerns this phenomenon. Barham and Keller [10] suggested that there were circumferential cracks in spherulites which had been crystallized at a temperature above ca. 120°C. What is important is that there may be several points prone to degradation in the crystal and that these degradable points will be sources of carbon. If the quantity of carbon supply to a point is enough for the formation of a colony, the degrading bacterium may form a colony at this point and, consequently, a spherical hole. On the contrary, if the carbon supply is insufficient owing to slow degradation, such as in the case of Sample 130, no spherical colony will be formed.

On the basis of the above results, Fig. 13 shows schematically the presumed two types of degradation processes whereby fundamentally the entire surface of the amorphous regions may be eroded by enzymatic action, and at specific points, dictated by the crystal structure, the degrading bacterium may colonize to form spherical holes.

CONCLUSION

This report describes an investigation of the effects of crystal structure on the microbial degradation of PHB. It was found that the development of crystallinity evidently depressed the microbial degradability of PHB. SEM observations suggest that the microbial degradation proceeded in at least two manners. One was preferential degradation of amorphous regions leaving the crystalline lamellae intact, which is considered to be a homogeneous enzymatic degradation over the sample surface by diffused extracellular depolymerase. The

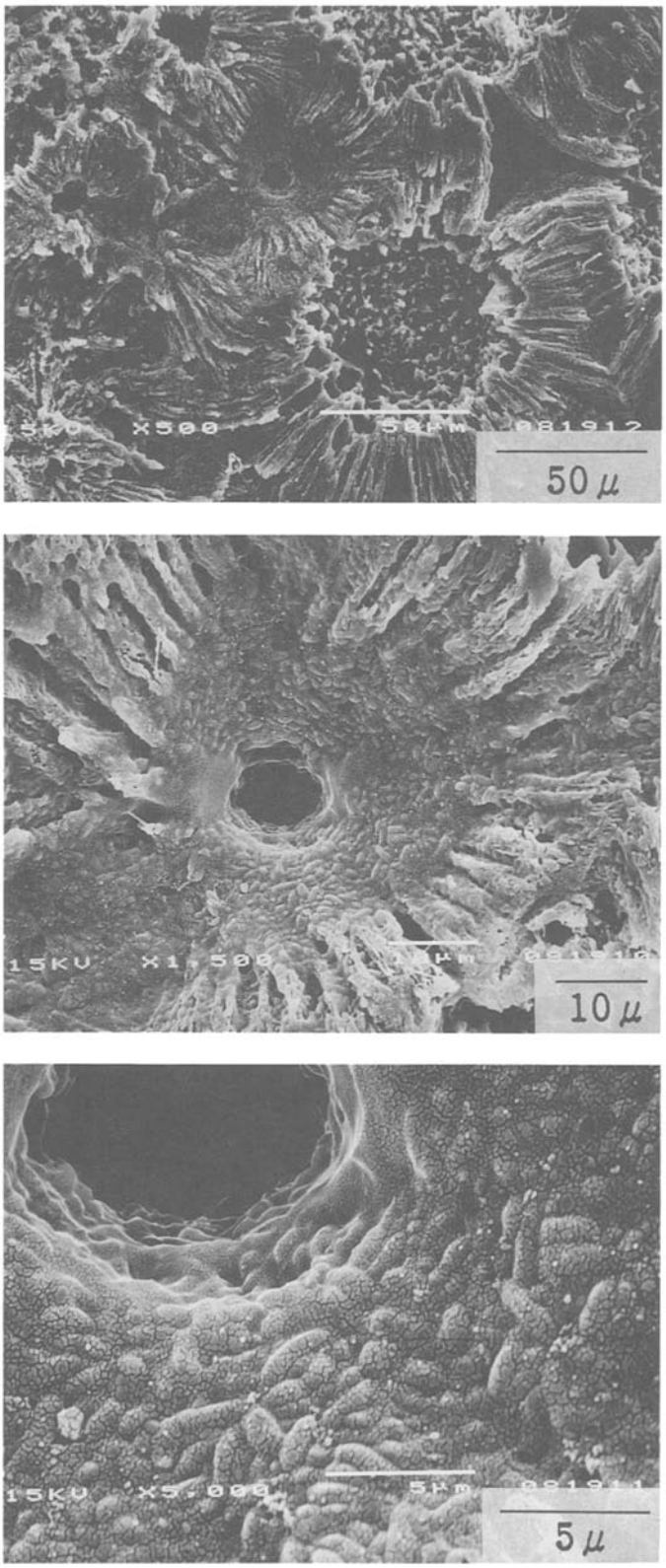
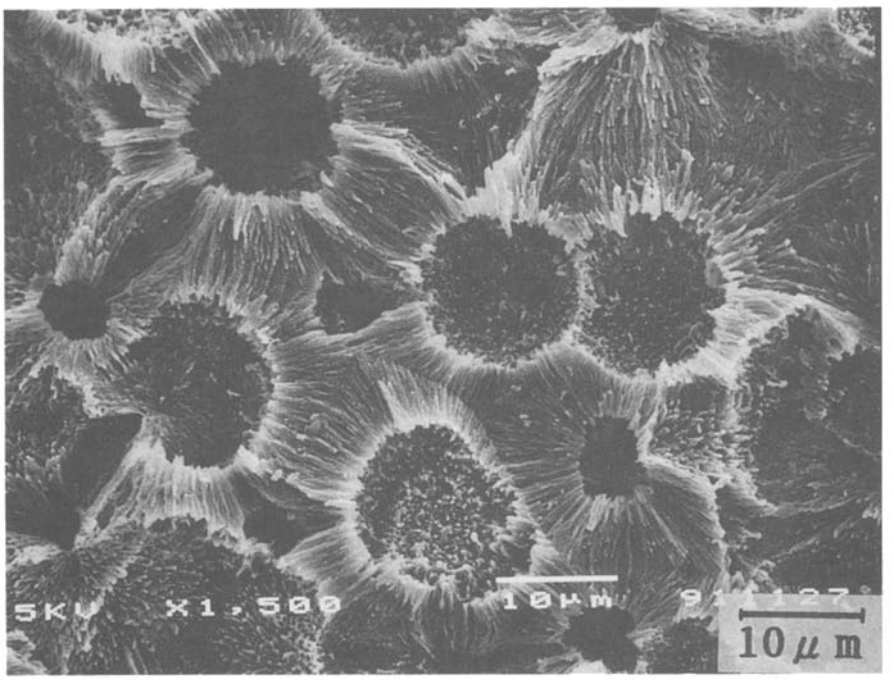
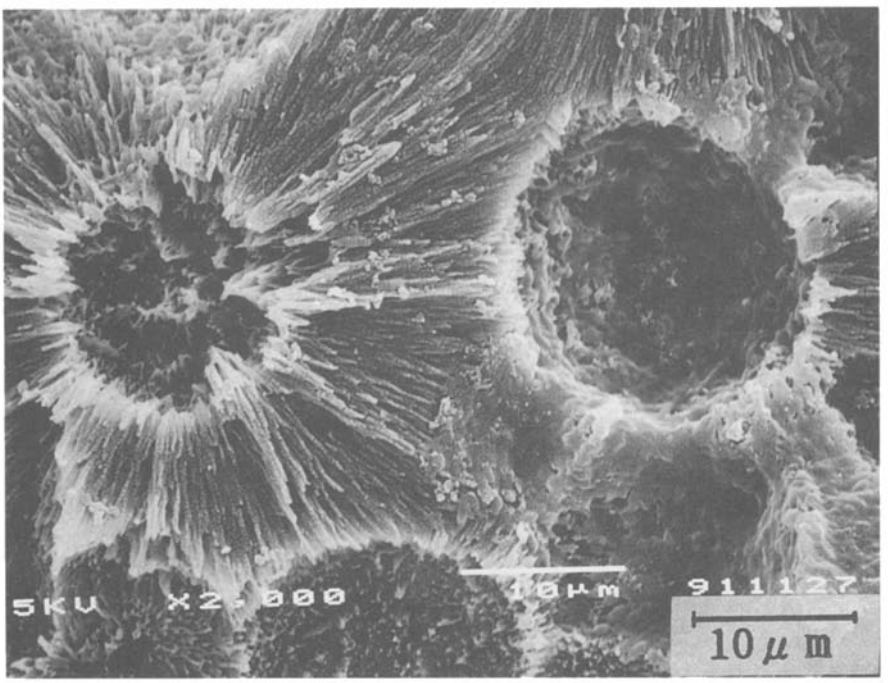


Fig. 11. SEMs of PHB-degrading bacteria: strain SC-17 in the process of degrading PHB Sample 60 after cultivation at 30°C for 72 h. (a) ×500; (b) ×1500; (c) ×5000—all parts reduced 10% for reproduction.



(a)



(b)

Fig. 12. SEMs of the spherical holes formed at the center of spherulites and on the boundary lines between spherulites after cultivation at 30°C for 72 h. (a) Sample −80; (b) Sample 0.

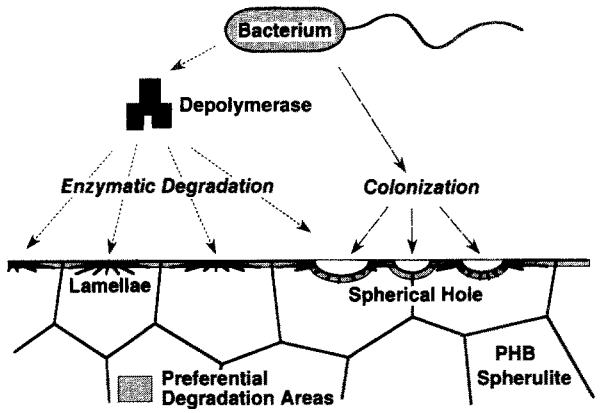


Fig. 13. Scheme of the presumed two manners of microbial degradation of crystallized PHB surface.

other was nonpreferential spherical degradation on the film surface. The SEMs indicated that the spherical holes resulted from colonization by the degrading bacteria. The holes varied in size and number with the change in crystal structure. Therefore, it was considered that the crystal structure of PHB also influenced the physiological behavior of degrading bacteria on the PHB surface.

REFERENCES

1. W. J. Cook, J. A. Cameron, J. P. Bell, and S. J. Huang (1981) *J. Polym. Sci., Polym. Lett. Ed.* **19,** 159–165.
2. C. V. Benedict, W. J. Cook, P. Jarrett, J. A. Cameron, S. J. Huang, and J. P. Bell (1983) *J. Appl. Polym. Sci.* **28,** 327–334.
3. P. Jarrett, C. V. Benedict, J. P. Bell, J. A. Cameron, and S. J. Huang (1984) in S. W. Shalaby, A. S. Hoffman, B. D. Ratner, and T. A. Horbett (Eds.), *Polymers as Biomaterials*, Plenum Press, New York, pp. 181–192.
4. R. Alper, D. G. Lundgren, R. H. Marchessault, and W. A. Cote (1963) *Biopolymers* **1,** 545–556.
5. J. Cornibert and R. H. Marchessault (1972) *J. Mol. Biol.* **71,** 735–756.
6. M. Yokouchi, Y. Chatani, H. Tadokoro, K. Teranishi, and H. Tani (1973) *Polymer* **14,** 267–272.
7. R. H. Marchessault, S. Coulombe, H. Morikawa, K. Okamura, and J. F. Revol (1981) *Can. J. Chem.* **59,** 38–44.
8. P. J. Barham, A. Keller, E. L. Otun, and P. A. Holmes (1984) *J. Mater. Sci.* **19,** 2781–2794.
9. P. J. Barham (1984) *J. Mater. Sci.* **19,** 3826–3834.
10. P. J. Barham and A. Keller (1986) *J. Polym. Sci., Polym. Phys. Ed.* **24,** 69–77.
11. M. Scandola, M. Pizzoli, G. Ceccorulli, A. Cesaro, S. Paoletti, and L. Navarini (1988) *Int. J. Biol. Macromol.* **10,** 373–377.
12. J. M. Merrick and M. Doudoroff (1964) *J. Bacteriol.* **88**(1), 60–71.
13. F. P. Delafield, M. Doudoroff, N. J. Palleroni, C. J. Lusty, and R. Contopoulos (1965) *J. Bacteriol.* **90**(5), 1455–1466.
14. C. J. Lusty and M. Doudoroff (1966) *Biochemistry* **56,** 960–965.
15. H. Tanaka, K. Tonomura, and A. Kamibayashi (1976) *Nippon Nougeikagaku Kaishi* **50**(9), 431–436.
16. T. Tanio, T. Fukui, Y. Shirakura, T. Saito, K. Tomita, T. Kaiho, and S. Masamune (1982) *Eur. J. Biochem.* **124,** 71–77.
17. K. Nakayama, T. Saito, T. Fukui, Y. Shirakura, and K. Tomita (1985) *Biochim. Biophys. Acta* **827,** 63–72.
18. Y. Shirakura, T. Fukui, T. Saito, Y. Okamoto, T. Narikawa, K. Koide, K. Tomita, T. Takemasa, and S. Masamune (1986) *Biochim. Biophys. Acta* **880,** 46–53.
19. T. Fukui, T. Narikawa, K. Miwa, Y. Shirakura, T. Saito, and K. Tomita (1988) *Biochim. Biophys. Acta* **952,** 164–171.
20. D. F. Gilmore, R. C. Fuller, and R. Lenz (1990) in S. A. Barenberg, J. L. Brash, R. Narayan, and A. E. Redpath (Eds.), *Degradable Materials*, CRC Press, Florida, pp. 481–514.
21. P. H. Janssen and C. G. Harfoot (1990) *Arch. Microbiol.* **154,** 253–259.
22. J. M. Merrick, D. G. Lundgren, and R. M. Pfister (1965) *J. Bacteriol.* **89**(1), 234–239.
23. Y. Kawaguchi and Y. Doi (1990) *FEMS Microbiol. Lett.* **70,** 151–156.
24. S. J. Holland, A. M. Jolly, M. Yasin, and B. J. Tighe (1987) *Biomaterials* **8,** 289–295.
25. M. Kunioka, A. Tamaki, and Y. Doi (1989) *Macromolecules* **22,** 694–697.
26. Y. Doi, Y. Kanesawa, and M. Kunioka (1990) *Macromolecules* **23,** 26–31.
27. H. Nishida and Y. Tokiwa, *J. Appl. Polym. Sci.* (submitted).
28. S. Akita, Y. Einaga, Y. Miyaki, and H. Fujita (1976) *Macromolecules* **9**(5), 774–780.
29. Y. Tsunoda, I. Aishima, and K. Katayama (1959) *Kobunsi Kagaku* **16**(172), 491–494.
30. N. Grassie, E. J. Murray, and P. A. Holmes (1984) *Poly. Deg. Stab.* **6,** 95–103.
31. Y. Doi, Y. Kanesawa, Y. Kawaguchi, and M. Kunioka (1989) *Makromol. Chem., Rapid Commun.* **10,** 227–230.
32. C. E. Zobell (1943) *J. Bacteriol.* **46,** 39–56.
33. R. Hattori, T. Hattori, and C. Furusaka (1972) *J. Gen. Appl. Microbiol.* **18,** 271–283.