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Degradation of Polyvinyl Alcohol by *Brevibacillus laterosporus*: Metabolic Pathway of Polyvinyl Alcohol to Acetate

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Abstract Approximately 0.1 mg/ml of polyvinyl alcohol (PVA) was degraded by the growing cell, *Brevibacillus laterosporus*, for 30 h, and 0.2 mg/ml of PVA was degraded by the cell-free extract that was isolated from *Brevibacillus laterosporus*. Approximately 0.29 µg/ml of acetic acid was produced from PVA by using the cell-free extract as a catalyst for 40 min. V_{max} and K_m value of purified PAV-degradation enzyme was 3.75 g/l and 2.75 g/l/min in reaction without EDTA and 3.99 g/l and 2.98 g/l/min in reaction with EDTA, respectively. Molecular weight of the purified enzyme determined by SDS-PAGE was 63,000 Da. Alcohol dehydrogenase and aldehyde dehydrogenase activities were qualitatively detected on a native acrylamide gel by an active staining method, indicating the existence of the metabolic pathway to use PVA as a substrate.

Key word: Polyvinyl alcohol, PVA-degrading enzyme, metabolic pathway, *Brevibacillus laterosporus*, aldehyde dehydrogenase, alcohol dehydrogenase

Some soil bacteria are known to degrade various natural polymers [6, 19], and assimilate polyvinyl alcohol (PVA) or a water-soluble synthetic polymer [13, 21]. An enzyme activity responsible for the degradation of PVA molecules has been found in a culture broth of a bacterial culture grown on a minimal medium containing PVA as a sole source of carbon and energy [22]. Polyvinyl alcohol (PVA) has been applied to the dye industry for activating the coloring reaction of cloth. PVA biodegradation has been shown to be caused by oxidation, in which a two-enzyme catalyzed oxidation process breaks the carbon backbone of the polymer [22]. The enzymes responsible for cleavage of the polymer have been identified as oxidases [4, 20] or hydrolases [8]. A group of organisms that utilize these enzyme systems have been shown to degrade PVA from a variety of environments [8], but the degradation process is slow and different from each other in most cases [5].

PVA has been reported as a biodegradable compound [18] but it is difficult to be degraded by bacteria without any pretreatment [3]. Shimao *et al.* [14, 15], and Sakazawa *et al.* [13] reported that the PVA can be oxidized by a symbiotic mixed culture of *Pseudomonas* sp., and that culture conditions can influence the efficiency of degradation. Sakai *et al.* [11] reported that a low molecular carboxyl-PVA was produced from PVA by catalysis of PVA-oxidase, and Sakai *et al.* [12] reported that hydrogen peroxide was produced from PVA by catalysis of secondary alcohol oxidase. Shimao *et al.* [17] reported that PVA degradation by *Pseudomonas* sp. was coupled to the bacterial respiration, and it was dependant on respiration enzymes of quinone and cytochrome.

In this study, a new bacterium was isolated from soil based on petroleum-degrading property, and a catalytic activity for PVA-degradation was confirmed. An attempt was made to describe the purification and characterization of the PVA-degrading enzyme and the mechanism of the enzyme to catalyze the oxidation of PVA molecules to acetate.

MATERIALS AND METHODS

Chemicals

Chemicals used in all tests were purchased from Sigma (St. Louis, U.S.A.) and Aldrich (Milwaukee, U.S.A.).

Microorganism and Growth Conditions

Brevibacillus laterosporus was isolated from petroleum-contaminated soil with a minimal medium of BDH (0.5 g/l of KH_2PO_4, 1.0 g/l of NH_4Cl, 0.1 g/l of Yeast Extract and 3 ml/l of trace mineral solution, pH 7.1 before sterilization; Trace mineral: 0.5 g/l $MnSO_4$, 0.5 g/l $MgSO_4$, 1.0 g/l $CaCl_2$, 10 g/l NaCl, 2.0 g/l KNO_3, 0.1 g/l $NiCl_2$, 0.1 g/l $CoCl_2$, 0.1 g/l $SeSO_4$, 0.1 g/l WSO_4, and 0.1 g/l $MoSO_4$) with 5 g/l of

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PVA, that was identified with the API-50 CHB identification kit (bioMériux sa, Lyon, France). The identity of *Brevibacillus laterosporus* was checked again with the automatic API identification system. This system can continuously test the different metabolic products, the variable biochemical reaction, the variable substrate consumption, and the increase of biomass during bacterial growth. From these information, bacterial species can be identified with an accuracy of 99%.

The pure culture was suspended in 25% glycerol and then frozen at -85°C for maintenance, and used throughout this study. Mass cultivation was carried out with 4 l of BDH medium in a 5-l fermenter (Korea Fermenter co. Seoul, Korea) at 25°C for 24 h. Aeration rates were controlled at 0.5 l/min, and the pH was automatically controlled at 7.0.

Electron Microscopy (SEM)

SEM photographs were obtained by using Lee's method [9].

PVA Assay

A solution containing PVA (Sigma LALLS type, average MW, 30,000–70,000 Da) and all other solutions were prepared with double distilled water and autoclaved at 121°C for 20 min. PVA degradation activity was assayed by determining the amount of PVA concentration in bacterial culture or in enzyme reaction. The concentration of PVA was determined by a modified colorimetric technique described by Bugada and Rudin [1]. A 100 ml sample of the PVA solution or bacterial culture with PVA was diluted to a volume of 10 ml, and then 7 ml of the reaction agent, which was made from 5 ml of 4% boric acid and 2 ml of I_2 and KI (1.27 g of I_2 and 25 g of KI in 1 l) just before use, was added to the PVA solution. The solutions were equilibrated for 5 min, and then diluted to make up a 25 ml volume, and analyzed at 690 nm. All measurements were performed in triplicate.

Isolation and Concentration of Bacteria-Free Culture

For obtaining extra-cellular enzyme, bacterial cell was removed from the culture by centrifugation at 8,000 ×g and 4°C for 30 min. The supernatant was concentrated 100 times by using an ultra-filtrate system with YM-10 membrane (Amicon, Beverly, MA, U.S.A.).

Preparation of Cell-Free Extract

Twenty-four h-cultivated cells were centrifuged at 8,000 ×g and 4°C for 30 min, and then washed twice with 25 mM phosphate buffer (pH 7.0). The harvested cells were pretreated with 3 mg/ml of lysozyme at 4°C for 90 min, and then disrupted by an ultrasonicator at 0°C for 10 min. Cell debris was discarded by centrifugation at 10,000 ×g and 4°C for 40 min, and the supernatant was centrifuged once more under the same condition to obtain a cell-free extract.

PVA-Degradation Activity of Bacterial Cell on Agar Plate

The bacterial cells grown on BDH broth was appropriately diluted and spread on BDH agar plate, and subsequently, the isolated colony was incubated at 25°C for 48 h. After incubation at 25°C for 30 min, 5 ml of the iodine reaction agent was poured on the agar plate containing bacterial colony and the color change around the colony was then compared.

Purification of PVA-Degradation Enzyme from Bacterial Culture

The PVA-degradation enzyme excreted from the bacterial cell to the culture was concentrated by using the ultra-filtration system with YM-10 membrane (Amicon, Beverly, MA, U.S.A.). The enzyme was purified by ion-exchange chromatography with DEAE-cellulose (Bio-Rad), gel filtration with Biogel P-30 and P-10, and then FPLC (Pharmacia, Sweden) with a Superose 6 HR column. The molecular weight of purified enzyme was determined by SDS-PAGE. The molecular weight was found to be 63,000 Da by comparing with the molecular weight markers (Bio-Rad, Richmond, CA, U.S.A.).

Active Staining of Alcohol Dehydrogenase and Aldehyde Dehydrogenase

Native gel electrophoresis was used for separation of enzymes. Cell-free extract was resolved by using a native polyacrylamide gel electrophoresis, while the gel was soaked in 50 mM Tris-HCl buffer (pH 8.0) containing substrate (10 mg/ml of PVA or 10 mg/ml of acetaldehyde), NAD^+ (2 mg/ml), phenazine methosulfate (0.5 mg/ml), and methyl thiazolyl tetrazolium (1 mg/ml). Then, it was incubated at 25°C for 30 min. The NADH is reduced by coupling with oxidation of the PVA or acetaldehyde by the enzymes located in the acrylamide gel, phenazine methosulfate and methyl thiazolyl tetrazolium, which is converted to formazan (dark blue) and subsequently oxidizes NADH to NAD^+ [23].

Acetate Production from PVA by Cell-Free Extract

The reaction was started by adding the cell-free extract to 1 mg/ml of PVA solution in phosphate buffer (50 mM, pH 7.0) at 25°C. The amount of reaction mixture was adjusted to 50 ml, and 1 ml of the sample was taken from the reaction mixture at 10 min intervals. One-tenth ml of 1 N HCl was added to the sample, and the sample was then centrifuged at 12,000 ×g for 30 min to remove cell debris and suspended solid, and the supernatant was used for gas chromatographic analysis.

Acetate Analysis

Acetate was analyzed by using gas chromatography equipped with both flame-ionized detector and capillary column (Varian Stabilwax, 30 m×0.25 mm). Nitrogen was

used as the carrier gas (8 ml/min). The temperatures of injector, column, and detector were 180°C, 100–250°C, and 250°C, respectively.

Determination of K_m and V_{max}

The Lineweaver-Burk method was used for calculation of K_m and V_{max} values [10].

RESULTS AND DISCUSSION

The bacterial strain used in this experiment can grow in BDH medium with PVA as a sole carbon source. It excreted extracellular PVA-degrading enzyme which degraded the PVA (average molecular weight of PVA was 30,000–70,000 Da), which was too big to be transported through the bacterial membrane. As shown in Fig. 1, the color around the bacterial colony was not changed to a blue or brown by adding the reaction agent for coloring PVA. The PVA can be changed to a blue color by reaction with iodine and the blue color is converted to brown after incubation for 20 min. The significance of the result was that PVA was degraded by extracellular enzyme that was excreted by the test strain on the agar plate. This method was very useful for directly isolating PVA-degrading bacterial strain on agar plate using minimal medium with PVA. Generally, PVA-degrading bacteria have been isolated from soil or other habitats by using a test tube assay after colonies were isolated from the agar plate [16]. The growing cell in broth has a hyphae under a light microscope (data not shown), but under the electron microscope, the cells are seen to be connected to each other by a string-like material, as shown in Fig. 2. The morphology of the test strain was similar to *Bacillus* with an exception that it had a slime-like compound quite different from a *Bacillus*. In a broth cultivation, the PVA concentration was assayed with iodine agent in the supernatant of bacterial culture after centrifugation. As show in Fig. 3, approximately 0.12 mg/ml of PVA was degraded by growing cells of the test strain for 30 h, and bacterial growth was proportional to the degree of PVA degradation in the broth cultivation. Since extracellular PVA degrading enzyme had to be purified from the bacterial culture but not from the extract, a cell-free culture was isolated from a 24-h-old culture using a BDH medium, and then concentrated. The concentrated bacterial-free culture was used as a catalyst for determining the PVA-degradation activity. As shown in Fig. 4, K_m and V_{max} were at 2.75 g/l and 3.75 g/l/min, respectively, without EDTA treatment, and at 2.98 g/l and 3.99 g/l/min with EDTA treatment, respectively. Only a slight increase of K_m and a little decrease of V_{max} by EDTA treatment suggested that the PVA-degrading

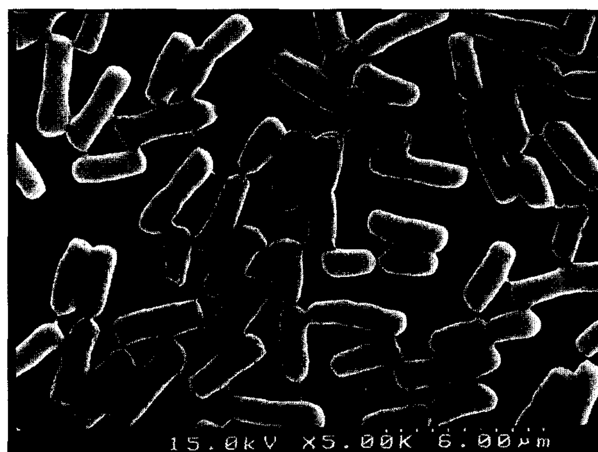

Fig. 2. Electron microscopy photograph of *Brevibacillus laterosporus*.
Each cell was connected to each other with a mucous-like material.

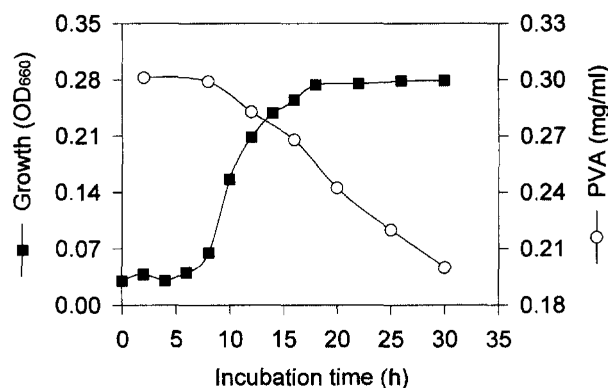

Fig. 3. *Brevibacillus laterosporus* grown on minimal medium with PVA as a sole carbon source. About 0.1 mg/ml of PVA was degraded for 30 min.
The PVA degradation was proportional to bacterial growth.

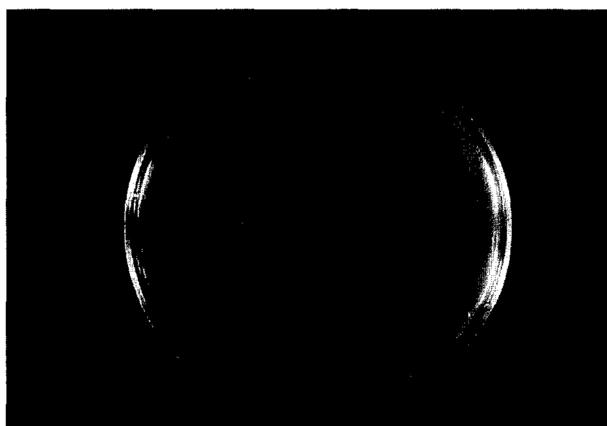

Fig. 1. Photograph of agar plate with PVA on which *Brevibacillus laterosporus* grew, and PVA was degraded by growing cells.
The white spot around a colony indicates that PVA was degraded. PVA forms a blue or dark brown color by reacting with iodine.

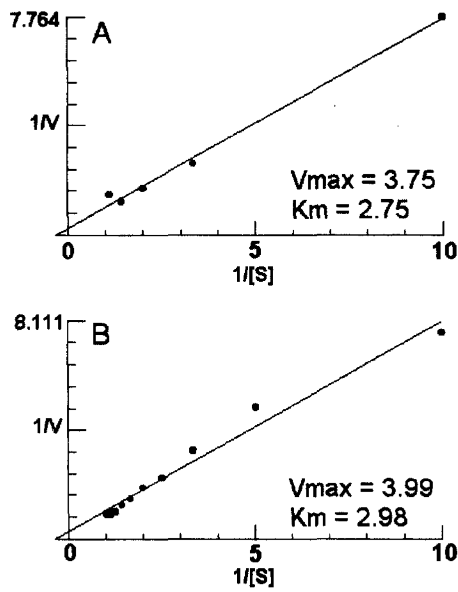

Fig. 4. Determination of kinetic constants by Lineweaver-Burk graphical method.
Non-EDTA-treated (A) and EDTA-treated enzymes were used to confirm metal ion effect on enzyme activity. These results show that the PVA-degrading enzyme did not require any metal ion for activity.

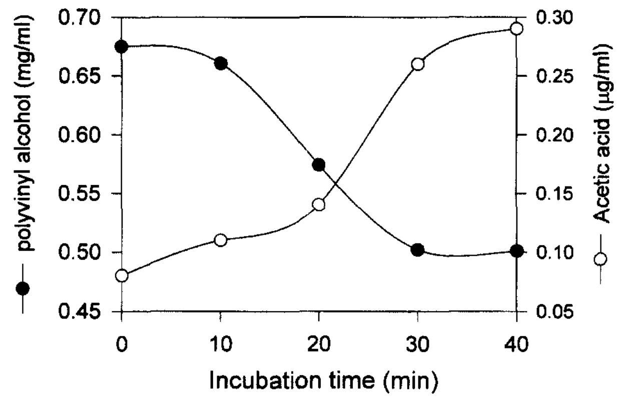

Fig. 5. Acetate production from PVA by cell-free extract.
Final protein concentration of cell-free extract was 0.13 mg/ml and reaction was performed in 50 mM of phosphate buffer with final 0.68 mg/ml of PVA at 25°C.

enzyme did not require cofactors such as metal ion for its activity and for its stability. Some enzymes that biodegrade polymer, or isomerase, have been reported to have metal ion as a cofactor [2, 7], and their activities are inhibited by EDTA treatment. From these results, the PVA-degradation enzyme described in this study was confirmed to not have metal ion as a cofactor. For confirmation of metal ion effects on the enzyme activity, Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, and Ca^{2+} were added to the enzyme preparation that was treated with EDTA (data not shown). In these tests, the enzyme activity was never increased by the addition of metal ions. The K_m value of PVA-degradation enzyme was also calculated by using Michaelis-Menten's equation, and the value was 2.58 g/l with EDTA treatment and 2.61 g/l without EDTA treatment, respectively, which were quite similar. To determine the metabolic pathway, a final product from biodegradation of PVA by a cell-free extract was analyzed. As shown in Fig. 5, the final product from PVA biodegradation was acetate, and the acetate concentration analyzed by GC was confirmed to increase in proportion to the time of treatment, as shown in Fig. 6. The acetate concentration produced by PVA biodegradation was 10 times lower than the amount of PVA consumed,

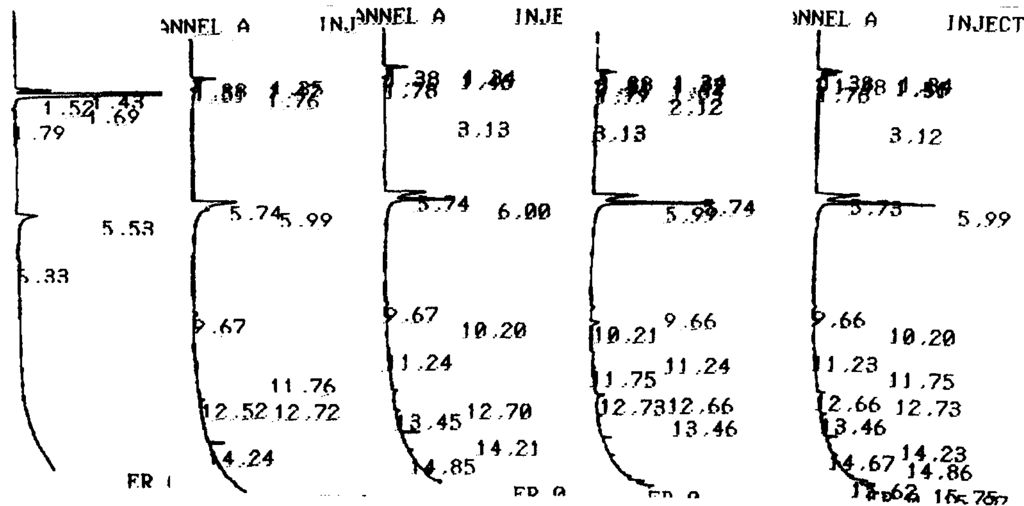

Fig. 6. Chromatogram of acetate analyzed by using gas chromatography.
Each chromatogram was obtained from analysis of sample isolated from enzyme reaction with PVA at a 10-min interval. A peak at retention time 5.99 (6.00) indicates acetate that increased in proportional amount to the reaction time.

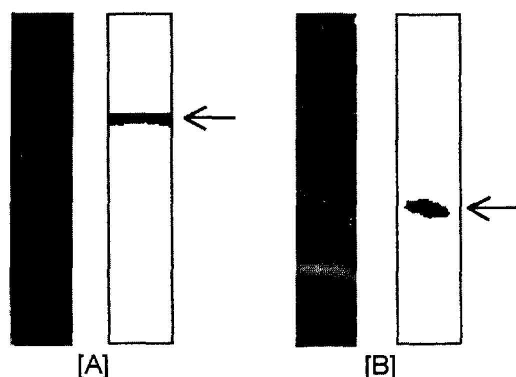

Fig. 7. Active staining of acetaldehyde dehydrogenase (A) and alcohol dehydrogenase (B), which were separated by native acrylamide gel electrophoresis.

The arrow-marked bands show a possibility that aldehyde and alcohol may be a metabolic intermediate and substrate, respectively, in metabolism of *Brevibacillus laterosporus* growing on PVA. The right side figure of each photo is a depiction of the band, since the photo is not clear.

suggesting that PVA degradation rate was higher than the acetate production rate *in vitro*.

For tracing the PVA degradation pathway in cellular metabolism, acetaldehyde dehydrogenase and alcohol dehydrogenase activities were analyzed qualitatively by an active staining of the native electrophoresis gel. As shown in Fig. 7, acetaldehyde dehydrogenase (A) and alcohol dehydrogenase (B) bands were observed on the acrylamide gel. Based on the result, the metabolic pathway can be proposed as shown in Fig. 8. It is quite possible that PVA hydrolyzed to ethanol [8], and acetic acid might have been produced via ethanol and acetaldehyde. It was confirmed that the test strain grew in a minimal medium with ethanol or acetate as a sole carbon source (data not shown). The acetate was confirmed to be a final product, as seen in Fig. 6. However, this is not a proven pathway but a proposed pathway, because it was confirmed that the enzyme activity did not metabolize the intermediate. In the future, an attempt will be made to analyze a possible metabolic intermediate for determining the real metabolic pathway. Although PVA is known as one of the variable industrial wastes, it can be a useful source for producing biodegradable polymers, such as polyhydroxy butyrate (PHB), through bacterial metabolism.

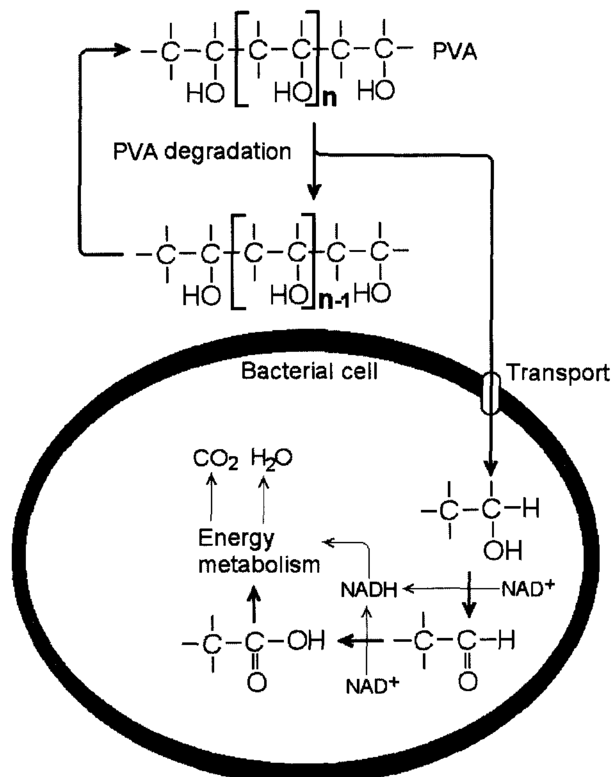

Fig. 8. Proposed pathway of the metabolic degradation of PVA to acetate.

Each metabolic step was determined by using alcohol dehydrogenase and acetaldehyde dehydrogenase.

REFERENCES

1. Bugada, D. C. and A. Rudin. 1985. Characterization of poly(vinyl alcohol). *J. Appl. Polym. Sci.* **30:** 4137–4147.
2. Cha, J. 1999. Contribution of second metal binding site for metal specificity of D-Xylose isomerase. *J. Microbiol. Biotechnol.* **9:** 757–763.
3. Daniel, M. L., R. J. Crawford, G. B. Y. Christie, and G. T. Lonergan. 1999. Enhanced degradation of polyvinyl alcohol by *Pycnoporus cinnabarinus* after pretreatment with Fentons reagent. *Appl. Environ. Microbiol.* **65:** 1798–1800.
4. Finch, C. A. 1992. *Polyvinyl Alcohol Development.* John Wiley and Sons Ltd., New York, NY, U.S.A.
5. Fukae, R., T. Fujii, M. Takeo, T. Yamamoto, T. Sato, Y. Maeda, and O. Sangen. 1994. Biodegradation of poly(vinyl alcohol) with high isotacticity. *Polym. J.* **26:** 1381–1386.
6. Jung, E. J., P. K. Shin, and H. K. Bae. 1999. Effects of temperature and compost conditions on the biodegradable polymers. *J. Microbiol. Biotechnol.* **9:** 464–468.
7. Jung, H. J., H. K. Kim, and J. I. Kim. 1999. Purification and characterization of Co^{2+}-activated extracellular metalloprotease from *Bacillus* sp. JH108. *J. Microbiol. Biotechnol.* **9:** 861–869.
8. Kawagoshi, Y. and M. Fujita. 1998. Purification and properties of the polyvinyl alcohol-degrading enzyme 2,4-pentanedione hydrolase obtained from *Pseudomonas vesicularis* var. povallyticus PH. *Water J. Microbiol. Biotechnol.* **14:** 95–100.
9. Lee, Y. N., J. H. Lee, H. J. Cho, E. J. Shin, J. W. Park, and J. H. Park. 1999. Characterization of Campylobacters newly isolated from swine gastric mucosa. *J. Microbiol. Biotechnol.* **9:** 778–783.
10. Price, N. C. and L. Stevens. 1989. *Fundamentals of Enzymology.* Second edition, Oxford Science Publication, U.K.

11. Sakai, K., M. Morita, N. Hamada, and Y. Watanabe. 1981. Purification and properties of oxidized poly(vinyl alcohol)-degrading enzyme. *Agric. Biol. Chem.* **45:** 63–71.
12. Sakai, K., N. Hamada, and Y. Watanabe. 1986. Degradation mechanism of poly(vinyl alcohol) by successive reactions of secondary alcohol oxidase and β-diketone hydrolase from *Pseudomonas* sp. *Agric. Biol. Chem.* **50:** 989–996.
13. Sakazawa, C., M. Shimao, Y. Taniguchi, and N. Kato. 1981. Symbiotic untilization of polyvinyl alcohol by mixed cultures. *Appl. Environ. Microbiol.* **41:** 261–267.
14. Shimao, M., Y. Taniguchi, S. Shikata, N. Kato, and C. Sakazawa. 1982. Production of polyvinyl alcohol oxidase by a symbiotic mixed culture. *Appl. Environ. Microbiol.* **44:** 28–32.
15. Shimao, M., I. Fukuta, N. Kato, and C. Sakazawa. 1984. Mixed continuous cultures of polyvinyl alcohol-utilizing symbionts *Pseudomonas putida* VM15A and *Pseudomonas* sp. Strain VM15C. *Appl. Environ. Microbiol.* **48:** 751–754.
16. Shimao, M., Y.Nishimura, N. Kato, and C. Sakazwa. 1985. Localization of polyvinyl alcohol oxidase produced by a bacterial symbiont, *Pseudomonas* sp. Strain VM15C. *Appl. Environ. Microbiol.* **49:** 8–10.
17. Shimao, M., S. Onishi, N. Kato, and C. Sakazawa. 1989. Pyrrologquinoline quinone-dependant cytochrome reduction in polyvinyl alcohol-degrading *Pseudomonas* sp. Strain VM15C. *Appl. Environ. Microbiol.* **55:** 275–278.
18. Shimao, M., I. Fujita, N. Kato, and C. Sakazawa. 1985. Enhancement of pyrroloquinoline quinone production and polyvinyl alcohol degradation in mixed continuous cultures of *Psedomonas putida* VM15A and *Pseudomonas* sp. Strain VM15C with mixed carbon sources. *Appl. Environ. Microbiol.* **49:** 1389–1391.
19. Shin, P. K. and E. J. Jung. 1999. Effects of various parameters on biodegradation of degradable polymers in soil. *J. Microbiol. Biotechnol.* **9:** 784–788.
20. Suzuki, T. and A. Tsuchii. 1983. Degradation of diketones by polyvinyl alcohol degrading enzyme produced by *Pseudomonas* species. *Proc. Biochem.* **18:** 13–16.
21. Suzuki, T. 1978. Oxidation of secondary alcohols by polyvinyl alcohol-degrading enzyme produced by Pseudomonas O-3. *Agric. Biol. Chem.* **42:** 1187–1194.
22. Watanabe, Y., M. Morita, M. Hamada, and Y. Tsujidaka. 1976. Studies on the polyvinyl alcohol-degrading enzyme. Part 1. Purification and properties of a polyvinyl alcohol-degrading enzyme produced by a strain of *Pseudomonas*. *Arch. Biochem. Biophys.* **174:** 573–579.
23. Werthamer, S., A. Freiber, and L. Amaral. 1973. Quantitation of lactate dehydrogenase isoenzyme patterns of the developing human fetus. *Clin. Chim. Acta* **45:** 5–11.