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A Thermophile Degrading Poly(Ethylene-*co*-Vinyl Alcohol)

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A thermophile, strain 29, degrading poly(ethylene-*co*-vinyl alcohol)(EVOH-68) film including 68 mol% of poly (vinyl alcohol) segment was isolated from 63 soil samples obtained from different locations by an enrichment culture technique at 60°C. The strain grew on EVOH-68. During the growth, the molecular weight and residual weight of EVOH-68 decreased, accompanied by an increase in dissolved total organic carbon concentration (TOC) in the medium. The strain was identified as a close neighboring species to *Bacillus pallidus*, which has an optimum growth temperature of around 58°C.

Key words: Thermophile, Poly(Ethylene-*co*-Vinyl Alcohol), Biodegradation, *Bacillus pallidus*

Recently, the production of synthetic polymers mainly for use as plastic materials has been steadily increasing, which results in the production of enormous waste, causing environmental pollution due to the resistance of synthetic polymers to biodegradation.

Under these circumstances, many research papers on the biodegradability of synthetic polymers have been published. It has been observed that many kinds of synthetic polymers hardly biodegrade while some of them biodegrade with relative ease. Aliphatic polyester belongs to the latter category, becoming the center of interest as an biodegradable material[4]. Polyethylene (PE), except for its oligomers with molecular weight of under one thousand, belongs to the former category[2,20]. Since polyolefins, such as PE, have a great majority of plastic materials which are widely used, it is important to give biodegradability to PE. From this viewpoint, some pretreatment[1,3,21] and additives[1,3,7] were found to be helpful to recognizable biodegradation of PE, and the biodegradation without them was reported to take a very long period of time, e.g. more than 10 years[3]. On the other hand, little attention has been given to the enhancement of biodegradability of PE by copolymerization with a suitable polymer.

The introduction of a biodegradable segment into a non-biodegradable polymer chain should be useful for the molecular design of a biodegradable polymer. Poly(vinyl alcohol) (PVA) segment can serve a purpose like this, because PVA is known to be degradable by some bacteria[10,14,15,17]. Matsumura *et al.*[8,9,12] reported an improvement in biodegradability of a group of poly (sodium carboxylate) by incorporating a PVA segment. However, they were water-soluble polymers, and not plastic materials but detergents.

The present authors are interested in the biodegradation of poly(ethylene-*co*-vinyl alcohol) (EVOH) which is produced as an engineering plastic in a high quantity. EVOH is regarded as a type of PE including a biodegradable PVA segment, but there are no papers discussing its biodegradation, excluding our preceding one[19].

The present work investigates the microorganisms that degrade EVOH of practical use. The authors have carried out screening of thermophiles degrading EVOH by an enrichment culture technique at 60°C. Recently, composting has been attracting interest as a method of waste treatment for plastic materials. Thermophiles degrading synthetic polymers should be useful as a compost seed, since thermophiles are known to play a leading role in compost fermentation. From this viewpoint, the authors[19] previously isolated a thermophile degrading EVOH and identified it as *Bacillus stearothermophilus*. As a plastic material two types of EVOH are common, that is, one (EVOH-56) including 56 mol% of PVA segment and another (EVOH-68) including 68 mol% of PVA segment. The isolated thermophile was able to degrade EVOH-56, but not EVOH-68. Recently, the authors have successfully isolated a new thermophile degrading EVOH-68, which are described here.

EVOH-68 consisting of 68 mol% of PVA segment with a number average chain length (Ln) of 3.0 and 32 mol% of PE segment with Ln of 1.4 was in the form of a 15-μm-thick film which had a number average molecular weight (Mn) of 17,100, a glass transition temperature (Tg) of 69°C and a

melting temperature of 183°C, supplied by Kuraray Ltd. (Osaka). EVOH-56 consisting of 56 mol % of PVA segment with *Ln* of 2.1 and 44 mol% of PE segment with *Ln* of 1.7 was in the form of a 15-μm-thick film which had *Mn* of 13,300, *Tg* of 55°C and a melting temperature of 165°C, also supplied by Kuraray Ltd. (Osaka). Prior to use, they were rinsed with 70% ethanol, and irradiated on both sides with ultraviolet light (Toshiba GL 15) for 30 min at a distance of 50 cm. Sixty-three soil samples were mainly collected from different locations in Kanagawa Prefecture, Japan. The composition of the basal medium (pH, 7.0) for enrichment culture was as follows: $(NH_4)_2SO_4$, 0.4%; K_2HPO_4, 0.2%; KH_2PO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; and yeast extract, 0.1%. Test tubes containing 5 ml of the medium sterilized at 121°C for 20 min in a high-pressure steam sterilizer and 25 mg of EVOH film cut into a square of 2 cm × 2 cm were inoculated with the above soil samples. Organisms were cultivated at 60°C for one week with rotary shaking at 120 rpm. For subculturing, this procedure was repeated twice. Cell growth was monitored by measurement of the absorbance at 660 nm using a Shimadzu UV 2200 spectrophotometer. Among the above soil samples, soil no. 29 exhibited a much higher growth rate on EVOH-68 than the others in the enrichment culture medium. One bacterial strain was isolated from this soil sample, and was designated as strain 29. This strain was investigated in detail as follows.

It was inoculated into the enrichment culture medium (100 ml in a 300-ml Erlenmeyer flask) including 500 mg of EVOH-68 film, and cultivated at 60°C for 20 days with rotary shaking at 120 rpm. The time course of assimilation was monitored by the level of absorbance at 660 nm and the

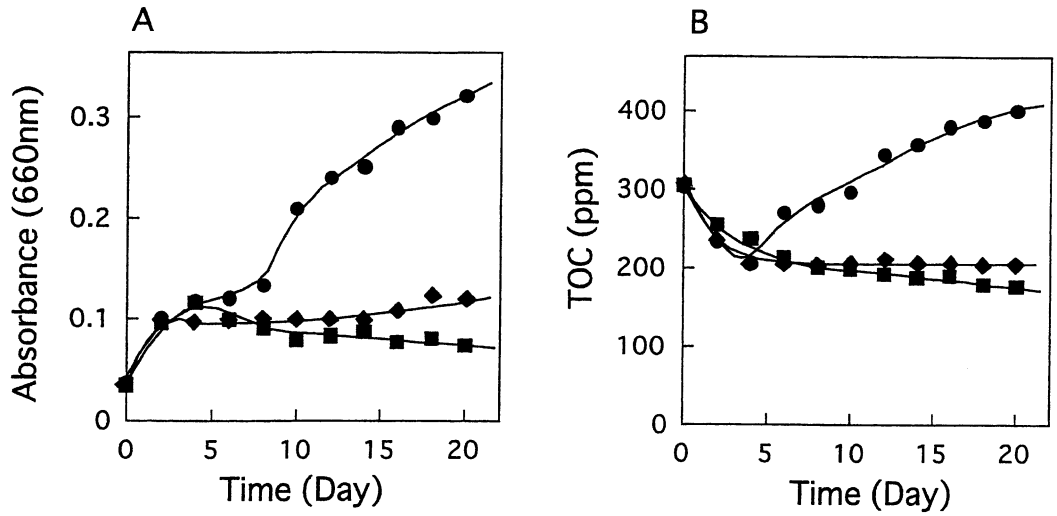

Fig. 1. Cultivation of strain 29 on EVOH at 60°C. (A) Absorbance of the culture broth at 660 nm. Symbols: ●, EVOH-68; ◆, EVOH-56; ■, control without EVOH. (B) TOC in the culture supernatant. Symbols: ●, EVOH-68; ◆, EVOH-56; ■, control without EVOH.

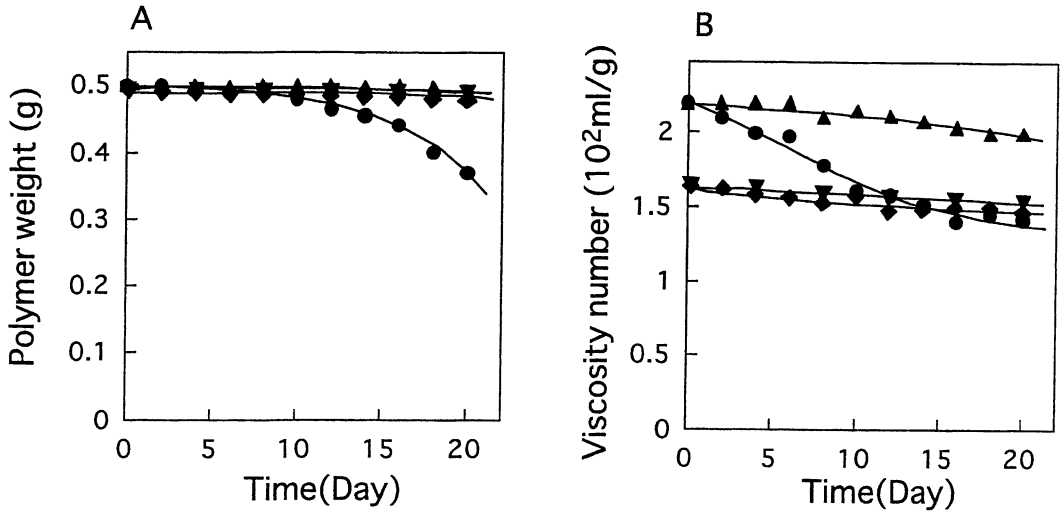

Fig. 2. Degradation of EVOH by strain 29. (A) Residual weight of EVOH. Symbols: ●, EVOH-68; ◆, EVOH-56; ▲▼, control without strain 29, EVOH-68(▲), EVOH-56(▼). (B) Viscosity number of residual EVOH. Symbols: ●, EVOH-68; ◆, EVOH-56; ▲▼, control without strain 29, EVOH-68(▲) , EVOH-56(▼). Definition of viscosity number is given in text.

dissolved total organic carbon concentration (TOC) for a culture broth. It was also monitored by the weight and the solution viscosity of residual EVOH-68 film which was picked up from the culture broth, washed with water, then with acetone, and dried. TOC was measured using a Shimadzu TOC 5000. As an index for the degradation of EVOH, its solution viscosity was measured at 30°C with an Ostwald viscometer. A phenol/1,1,1-trichloroethane (1 : 1) mixture was used as a solvent for EVOH, and the polymer concentration was 0.5 g/100 ml of the solvent. The results are shown in terms of viscosity number which is given by:

[(polymer solution flow time, s/solvent flow time, s) − 1]/(polymer concentration, g/100 ml).

The growth curve of strain 29 on EVOH-68, which is the increase in the absorbance at 660 nm, and the time course of TOC are shown in Figs. 1A and 1B, respectively. The cultivation was replicated three times and the mean values, whose mean coefficients of variation were below 20%, were plotted. The absorbance increased rapidly at the initial stage of growth and continued to increase after a short slack of period. TOC rapidly reached the minimum in the initial stage and then increased rapidly. On the other hand, the absorbance in the control (without EVOH) increased as much as in the run with EVOH-68 for the initial stage only and decreased gradually after reaching the peak. TOC in the control (without EVOH) decreased rapidly in the initial stage as much as in the run with EVOH-68, and then decreased gradually. These results suggest that initially the growth was dependent on a small amount of soluble carbon source in the medium and then later the microorganism utilized EVOH-68. The microbial degradation of EVOH-68 is assumed to result in a production of soluble compounds, which corresponds to the observed increase in TOC at a later stage. On the other hand, as for EVOH-56 in a similar cultivation, the strain showed only a little difference from the control without EVOH (Figs. 1A and 1B).

The degradation curves of EVOH-68 in the above cultivations are shown in Figs. 2A and 2B in terms of the decrease in weight and viscosity number of residual EVOH-68, respectively. The values are plotted in a similar manner in Figs. 1A and 1B. The viscosity number decreased noticeably from the initial stage of cultivation and the weight decreased in the later stage with a gradually increasing rate, which may suggest the occurrence of endogenous random degradation of EVOH-68. Meanwhile, in the absence of the strain, decreases in both weight and viscosity number of residual EVOH-68 were quite slight in comparison with the above, as also shown in the figures. Corresponding to the growth results, the strain did not show significant degradation of EVOH-56 (Figs. 2A and 2B).

The above findings lead to the conclusion that strain 29 has the capability of assimilating and degrading EVOH-68. The authors' previous strain degraded EVOH-56, but did not degrade EVOH-68, as mentioned in the introductory remarks. While PVA is soluble in water, EVOH is not. However, EVOH-56 has a *Tg* of 55°C under the cultivation temperature, which was inferred to be advantageous in

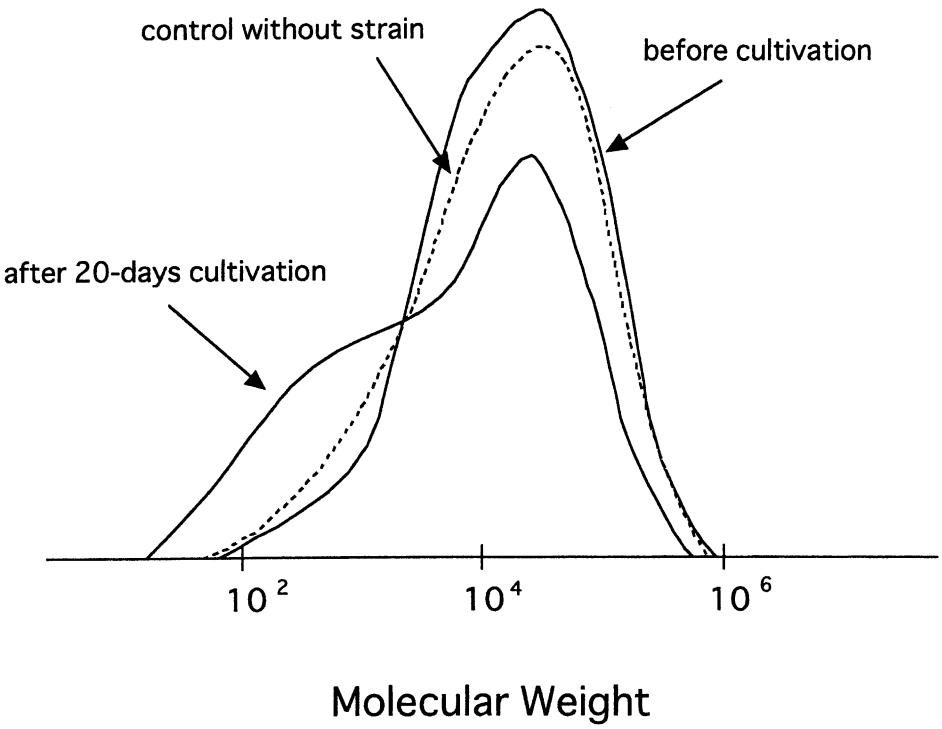

Fig. 3. GPC curves of EVOH-68 before and after 20-days cultivation of strain 29 at 60°C. The result for the control without the strain is also shown (dotted line). Column, Shim-pac GPC-80MD; Column temp., 40°C; Eluent, dimethylformamide; Detector, RI; Standard, Shodex Standard SM-105 (Polystyrene).

biodegradation from the polymer structure coming loose at the *T*g. On the other hand, EVOH-68 has the *T*g of 69°C over the cultivation temperature, which was considered to hinder microorganism from degrading polymers. In contrast, the present strain 29 did not degrade EVOH-56 but EVOH-68 contrary to the above consideration. For reference, the strain was applied to PVA degradation in a similar manner, where a PVA with *M*n of 34,500 was used. After 20-days cultivation, the absorbance of the culture broth at 660 nm increased from the initial value of 0.03 to 0.22 (control value without PVA of 0.07). PVA decreased in weight (g), which was determined according to the method of Finley[5], from the initial value of 0.500 to 0.365 (control value without the strain of 0.496) and in viscosity number (10^2 ml/g), which was measured in PVA of 0.5 g/water of 100 ml solution, from the initial value of 1.64 to 1.00 (control value without the strain of 1.62).

That is, this strain was capable of degrading and assimilating PVA as well as EVOH-68. The findings suggest the EVOH degradation by the strain should occur on the

Table 1. Microbiological properties of strain 29.

Cells	
Shape	Rod
Diameter (μm)	0.6 - 1.0
Length (μm)	3.5 - 5.5
Gram stain	+
Spores	+
Shape	Ellipsoidal
Location	Subterminal position
Sporangium	A little swollen
Motile	+
Aerobes	+
Catalase	+
Anaerobic growth	−
Voges-Proskauer test	−
pH in V-P broth	7.2
Acid from D-glucose	+
Gas from glucose	−
Hydrolysis of	
Gelatin	+
Starch	−
Utilization of	
Citrate	−
Propionate	+
Egg-yolk lecithinase	−
Reduction of nitrate	+
Growth at pH	
6.8	+
5.7	−
Growth in NaCl	
5 %	−
7 %	−
Growth at	
30 ℃	−
40 ℃	+
60 ℃	+
65 ℃	−
Mol % G + C	56

PVA segment in EVOH. From a difference in *L*n between EVOH-56 and -68, the strain may require *L*n of at least three PVA units for the degradation of EVOH. Matsumura *et al.*[8,9,12] reported that a PVA segment over 5–7 *L*n generally acts as a biodegradable segment in poly(sodium carboxylate-*co*-vinyl alcohol). As for EVOH-68, segments of 5–7 PVA units can be inferred to accompany the main segment of three PVA units in the proportion of 0.15–0.07 to one main segment, from the randomness of copolymerization of ethylene and vinyl acetate for the synthesis of EVOH. These additional segments may be utilized by the strain. They can be estimated to be quite fewer in EVOH-56 than in EVOH-68. In the experiments using model compounds, on the other hand, a chain of more than three PVA units having isotactic structure was reported to be effective in biodegradation, in contrast to an atactic structure of the same chain length[11]. Considering the atactic structure of PVA units in EVOH, it would be an interesting result if the present strain could utilize less of *L*n of the PVA segment than that in the above poly(sodium carboxylate) copolymer. Moreover, comparing the increase in absorbance at 660 nm between EVOH-68 and PVA after 20-days cultivation, the growth of the strain on EVOH-68 was superior to that on PVA, which may suggest that the presence of PE segment in EVOH-68 was advantageous for the growth and the strain recognized the PE segment. However, these details remain to be clarified.

In order to obtain further information about the change in the molecular weight of EVOH-68, gel permeation chromatography (GPC) curves, which was obtained using a Tosoh HLC-8220, were compared before and after 20-days cultivation of the strain. As shown in Fig. 3, the main peak decreased and a new shoulder appeared at the low molecular weight region. The control (without the strain) gave only a slight change in the GPC curve. Consequently, EVOH-68 is assumed to be first degraded to a low molecular weight EVOH and then assimilated by the strain.

As for the biodegradation of PVA, the PVA chain is regarded to be first oxidized, followed by hydrolytic cleavage by microbes to finally yield acetic acid[13]. Acetic acid generated from EVOH-68 in the culture supernatant was enzymatically analyzed according to our method[18]. In the supernatant of 20-days cultivation, acetic acid was detected in the concentration of 45.6 mg/l (control value without the strain of 9.0 mg/l), which suggests that EVOH-68 was degraded in the same mechanism as mentioned above. After 20-days cultivation, the weight of EVOH-68 decreased by 126.8 mg to yield 48.0 mg (both weights were the values after subtracting the controls) of the microbial cell which was collected by centrifugation at 9,400 × g for 20 min and dried at 60°C. From a carbon fraction in the structural unit of EVOH-68, the above decrement of 126.8 mg was evaluated to be a carbon amount of 79.4 mg, corresponding to 794 ppm in the culture broth. On the assumption that the carbon content of the microbial cell is estimated roughly at half of its weight, the cell weight of 48.0 mg could be evaluated to be a carbon amount of 24.0 mg, corresponding to 240 ppm in the culture broth, which suggests that the cell carbon occupied 240 ppm in 794 ppm carbon released from EVOH-68. One of the rest may be converted to soluble compounds, corresponding to TOC of 228 ppm (the value after subtracting the control) in the culture supernatant, and another to carbon dioxide. They would be comparable to each other.

The identification of strain 29 was carried out with the usual methods[6,16] and an analysis of 16S rDNA sequence. The strain was found to have the microbiological properties shown in Table 1. In summary, this strain was an aerobic, spore-forming, gram-positive, rod-shaped bacterium. It produced catalase and acid from glucose. It grew at a temperature between 40 - 60°C. These properties were consistent of thermophilic *Bacillus* sp. The sequence of the first 500 bp of 16S rDNA, analyzed by MicroSeq Bacterial 500 Library v.0023 (Applied Biosystems, CA), showed 99.8 % similarity to that of *B. pallidus*. The strain will be identified as a close neighboring species to *B. pallidus*. The optimum growth temperature was determined to be around 58°C using a temperature gradient stirred fermentor (Advantec TN 2148).

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