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Biodegradation of polyvinyl alcohol by a mixed microbial culture

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Abstract

A mixed culture capable of degrading 1 g l^{-1} polyvinyl alcohol (PVA) completely was screened from sludge samples at Pacific Textile Factory, Wuxi, China. This mixed culture had stronger capability of degrading PVA with low polymerization and high saponification than degrading PVA with high polymerization and low saponification. Inorganic nitrogen source was more suitable for the mixed culture to grow and degrade PVA than organic nitrogen source. Microorganisms and relative abundance of this mixed culture were explored by terminal restriction fragment length polymorphism (T-RFLP). Small PVA molecules were detected in cell extracts of the mixed culture. This indicated that PVA degradation in the mixed culture was in fact a combined action of extracellular and intracellular enzymes. Two strains producing extracellular PVA-degrading enzyme were isolated from the mixed culture. They could individually degrade PVA1799 with polymerization of 1700 from initial average molecular weight 112,981 to 98,827 Da and 84,803 Da, respectively. However, only small amount of PVA124 in polymerization of 400 could be degraded by these two strains.

Keywords: Polyvinyl alcohol (PVA); PVA-degrading enzyme; Mixed culture; T-RFLP; Biodegradation

1. Introduction

Polyvinyl alcohol (PVA), a water soluble synthetic polymer prepared by the hydrolysis of polyvinyl acetate, is widely used in adhesive, paper-coating, textile industries and as biodegradable polymer products. However, the dispose of PVA becomes one of the major pollutants in industrial wastewater, especially for textile industries. PVA is difficult to be degraded in natural environment and must be removed from the wastewater before discharge [1,2]. Single microorganism [3] and symbiotic or mixed culture [4–6] capable of degrading PVA have been identified. Several different enzyme systems to degrade PVA main-chains have been reported [7]. In these systems, the carbon–carbon linkage of PVA main-chains is cut first by the action of either an oxidase or a dehydrogenase, followed by a hydrolase or aldolase reaction [7]. However, the overall number of PVA-degrading microorganisms is rather limited in comparison to the widespread species capable of degrading aliphatic poly(esters), of both microbial and synthetic origin such as poly(hydroxyl-alkanoate)s (PHA's) and poly(ε-caprolactone) (PCL) [1]. Also, the presence of these microorganisms is restricted to rather peculiar environments, such as PVA-polluted textile or paper mill effluents [7].

In the paper, we present the study on the degradation behavior of PVA by an acclimated PVA-degrading heterogeneous microbial culture, and responsible microorganisms involved. The relative abundance of this mixed culture was explored by terminal restriction fragment length polymorphism (T-RFLP).

2. Materials and methods

2.1. PVA materials

Four types of PVA were used in this study. PVA1799 has a 1700 polymerization degree and 99.0% saponification degree. PVA1788 has a 1700 polymerization degree and 88% saponification degree. PVA0588 has a 500 polymerization degree and 88.0% saponification degree. PVA124 has a 400 polymerization degree and 99.0% saponification degree. These PVA were bought from Sichuan Vinyl factory (Mianyang, China).

2.2. Media

Basic medium (PVA1799–NH_4NO_3–yeast extract medium) contains (g l^{-1})—PVA1799: 1, NH_4NO_3: 1, yeast extract (Oxoid, UK): 1, K_2HPO_4: 1.6, $MgSO_4{\cdot}7H_2O$: 0.05, $CaCl_2$: 0.05, $FeSO_4{\cdot}7H_2O$: 0.02, and NaCl: 0.02. The pH was adjusted to 7.2. Screening medium is the same as basic medium, and 20 g l^{-1} of agar was added for solid medium.

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2.3. Microorganisms

Six soil samples and three activated sludge samples were collected from Pacific Textile Factory in Wuxi City, China. One gram of each these samples was added into 9 ml 0.8% (w/w) NaCl solution and shaken on a shaker for 10 min. Then 1 ml sample solution was inoculated into 250 ml flasks containing 30 ml screening medium, and cultured at 30 °C on a rotary shaker at 200 rev min^{-1} for 3 days. A sludge sample (the mixed culture) which could degrade 1 g l^{-1} PVA1799 completely was chosen for further study. Seven different bacterial strains were isolated from the mixed culture by dilution method and were named as strain Nos. 1–7.

2.4. Flask culture

The culture was carried out on a rotary shaker at 200 rev min^{-1} and 30 °C. The mixed culture or single strain was inoculated to 250 ml flasks containing 30 ml of the PVA medium and cultured till PVA is used up or PVA concentration is invariable.

2.5. Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP was performed according to the procedure reported by Femmell et al. [8]. Forward (5′-agagtttgatcmtggctcag-3′) and reverse (5′-cggtgtgtacaaggcccgggaacg-3′) primers capable of amplifying partial 16S rDNA from a broad range of taxonomically different bacterial strains were designed. PCR was performed in a total volume of 50 μl. The final reaction mixture contained 5 μl reaction buffer (500 mmol l^{-1} KCl, 1% Triton X-100, 100 mmol l^{-1} Tris–HCl, pH 9.0), 4 μl of 2.5 mol l^{-1} dNTP (Takara, Dalian, China), 4 μl of 25 mmol l^{-1} $MgCl_2$, 0.5 μl of 4.4 μmol l^{-1} forward and reserve primers, 0.5 μl of 5 U μl^{-1} *Taq* polymerase (Takara, Dalian, China), 0.5 μl of genomic DNA and 35.5 μl sterile distilled water. The denaturation step was conducted at 95 °C for 3 min, followed by primer annealing for 30 s at 54.5 °C and primer extension for 1.5 min at 72 °C. The final cycle was succeeded by an extension step at 72 °C for 10 min. The amplified PCR product was purified by a gel extraction mini kit (Watson Biotechnology Co., Shanghai, China).

2.6. Preparation of cell-free extract

Cells were harvested from flask culture by centrifugation at 8,000 × *g* for 10 min at 4 °C and washed with 10 mmol l^{-1} potassium phosphate buffer (pH 8.0) containing 1 mmol l^{-1} EDTA till PVA-iodine blue disappeared from cell surfaces. Washed cells were suspended in the same buffer to the original volume. The suspension was treated with an ultrasonic disintegrator (VCX 400; SONICS, USA) at 4 °C for 10 min and then centrifuged at 10,000 × *g* for 30 min (4 °C) to remove cell debris. The supernatant from centrifugation of flask culture was filtered through a 0.45 μm Millipore filter (YaDong Co. Ltd., Shanghai, China) to remove the cells completely and cell-free extract was obtained. The filtrate was dried before Infra Red (IR) analysis.

2.7. Analytical procedures

PVA both in culture broth and cell-free extract was measured according to the method reported by Finley [9]. A spectrophotometric method was developed for the determination of PVA. The method is based on the blue color produced by reaction of PVA with iodine in the presence of boric acid. Spectrophotometric measurement is conducted at the maximum absorption wavelength (690 nm), and Beer's law applies in the concentration range corresponding to 0–1 mg of PVA per 50 ml of solution.

Molecular weight distributions were determined by Gel Permeation Chromatograph (GPC) analysis by a 600E Waters chromatography (Waters, Milford, USA) equipped with 2410 Waters Refractive Index Detector (RI), M32 workstation and two Ultrahydrogel linear columns (Waters, Milford, USA) connected in series. Sample elution was carried out at 0.9 ml min^{-1} flow rate with 0.1 mol l^{-1} $NaNO_3$ at 45 °C. Monodisperse pullulan samples were used as standards. Before GPC analysis, 10 ml sample from liquid cultures was treated with 5 mg ml^{-1} CaO in order to remove phosphate, and filtrated through 0.2 μm PVDA filters. Then 5 μl of filtrate was analyzed.

IR analysis was carried out by Nicolet Nexus FT-IR spectrometer (Thermo Electron Co., USA). PVA used for IR spectrum analysis experiments was PVA1799.

Dry cell weight (DCW) was measured as follows. Four-milliliter culture broth was centrifuged at 10,000 × *g* for 10 min; the cells were washed twice with deionized water, and dried at 105 °C to constant weight.

3. Results and discussion

3.1. Effect of PVA type on biodegradation

Flask culture of the mixed culture was used to study its behavior of biodegrading different PVA. The results are given in Fig. 1. PVA124 with the lowest polymerization and

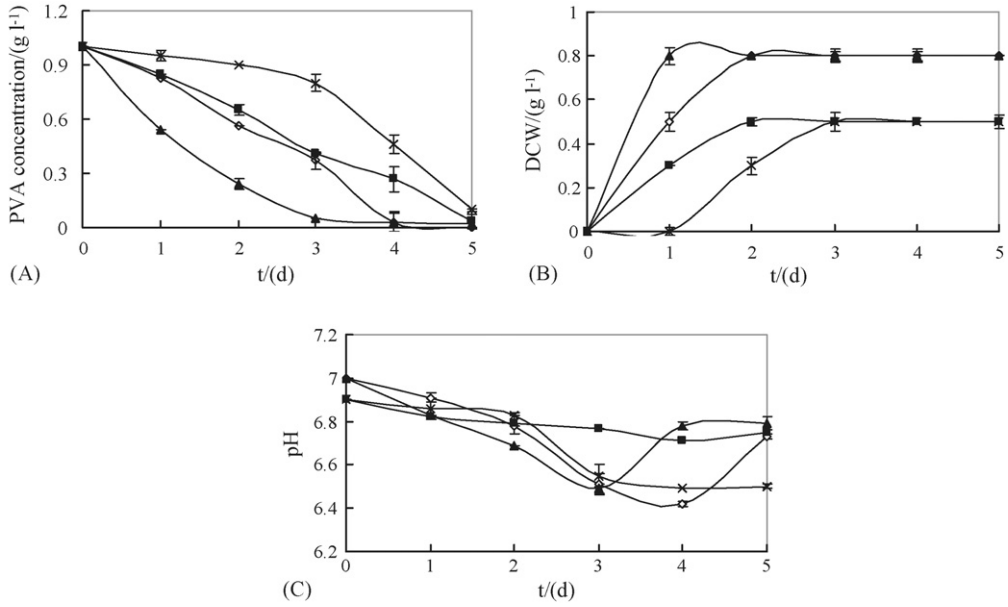

Fig. 1. PVA degradation (A) and cell growth (B) of the mixed culture. (◊) PVA1799; (■) PVA1788; (▲) PVA124; (×) PVA0588.

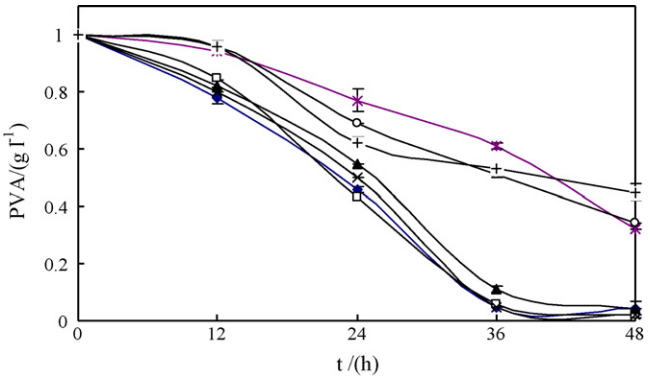

Fig. 2. Effect of nitrogen on PVA degradation by mixed culture. (♦) $NaNO_3$; (□) NH_4NO_3; (▲) $(NH_4)_2SO_4$; (×) urea; (✱) yeast extract; (◯) beef extract; (+) peptone.

the highest saponification could be degraded in 3 days, and PVA1799 with the highest polymerization and the highest saponification was completely degraded in 4 days. The complete degradation of PVA0588 and PVA1788 with lower degree of saponification took more than 5 days. It indicated that the degradation rate of PVA was dependent on the degree of saponification of PVA. More cell growth was observed when growth was on PVA124 and PVA1799 which were consistent with their quick biodegradation. And pH decreased during all the degradation courses due to the formation of some acidic intermediates.

3.2. Effect of nitrogen sources on biodegradation of PVA1799 by the mixed culture

Because PVA1799 was widely applied in textile industries, it was used in subsequent studies as model target. Mixed culture was cultivated in different nitrogen source (the mass concentration of N was kept 1 g l^{-1}). Fig. 2 shows that the use of inorganic nitrogen source was more suitable to degrade PVA than organic nitrogen source. The mixed culture could degrade PVA1799 completely in 36 h when inorganic nitrogen source was used in the medium. And three kinds of selected inorganic nitrogen sources showed similar effect on biodegradation of PVA1799.

3.3. Degradation of PVA1799 by the mixed culture

Fig. 3 shows the biodegradation course of PVA1799 (nitrogen source was 1 g l^{-1} NH_4NO_3) by the mixed culture. PVA1799 (g l^{-1}) could be degraded completely by mixed culture within 36 h. During this process, pH decreased from 7.0 to below 6. Table 1 shows the average molecular weight distribution

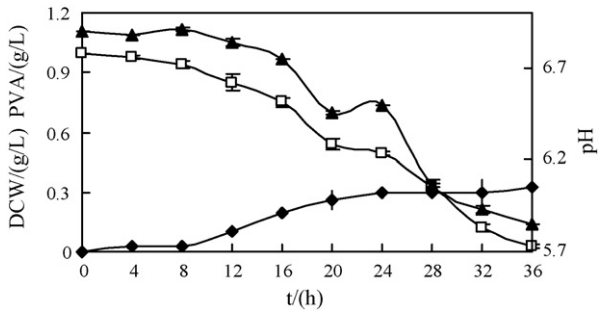

Fig. 3. Biodegradation course of PVA1799 by mixed culture. (▲) pH; (♦) DCW; (□) PVA concentration.

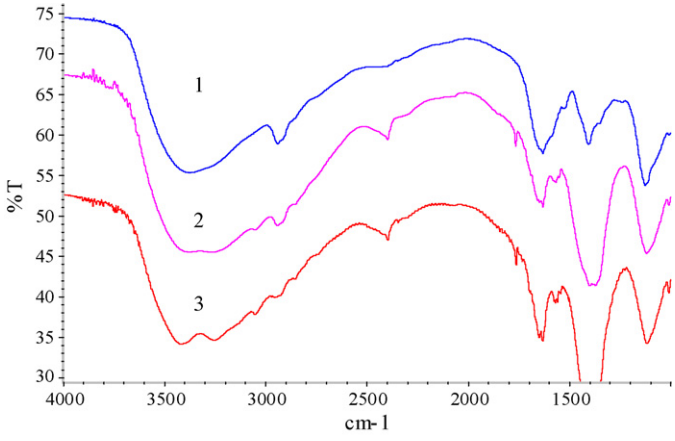

Fig. 4. IR spectra during PVA1799 degrading by mixed culture. (1) 0 h; (2) 16 h; (3) 36 h.

of PVA1799 decreased to zero at the end of the cultivation. IR spectra of PVA1799 at different culture time are shown in Fig. 4. Compared with IR spectrum of PVA at 0 h, during the degradation course, an absorption band at 1770 cm^{-1} and a zigzag absorption band at 3600–2500 cm^{-1} were observed, which attributed to carbonyl groups and carboxyl groups, respectively. In IR spectra of PVA at 16 and 36 h, an additional absorption at 1570 cm^{-1} was observed, probably resulting from the presence of carboxylic iron groups. These results indicated that carboxylic acids appeared during the PVA degradation course. These carboxylic acids (probably including aldehyde or ketone) were possible intermediate degradation products of PVA.

Seven bacteria were isolated from the mixed culture. Cultivating these seven bacteria together in PVA medium, PVA1799 could not be degraded completely anymore. When strains Nos. 1 and 7 were individually cultivated in PVA1799 and PVA124 medium, respectively, as shown in Tables 2 and 3 and Figs. 5 and 6, that strains Nos. 1 and 7 could degrade PVA1799

Table 1
Molecular weight distribution during degradation of PVA1799 by mixed culture

Culture time (h)	Migration time (min)	Molecular weight (Da)
0	16.113	112,981
12	16.567	92,724
24	16.867	71,955
36	16.567	0

Table 2
Molecular weight distribution during degradation of PVA by strain No. 1

Culture time (h)	Migration time (min)	Molecular weight (Da)
0 h in PVA1799 medium	16.113	112,981
72 h in PVA1799 medium	16.283	98,827
0 h in PVA124 medium	16.667	73,762
72 h in PVA124 medium	16.700	73,344

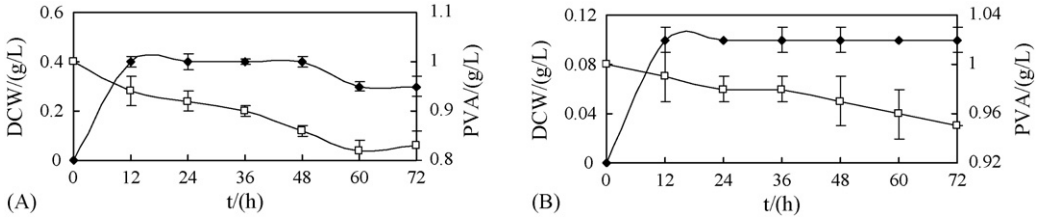

Fig. 5. Biodegradation of PVA1799 (A) and PVA124 (B) by strain No. 1. (♦) DCW and (□) PVA concentration.

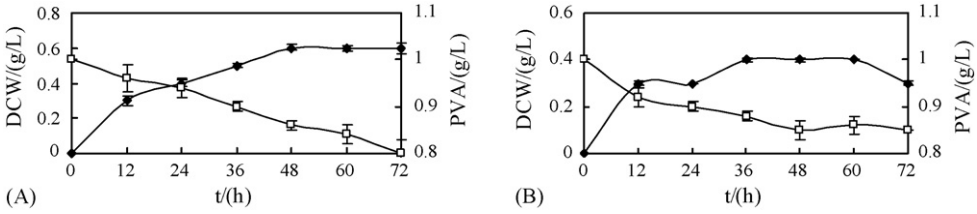

Fig. 6. Biodegradation of PVA1799 (A) and PVA124 (B) by strain No. 7. (♦) DCW and (□) PVA concentration.

Table 3
Molecular weight distribution during degradation of PVA by strain No. 7

Culture time (h)	Migration time (min)	Molecular weight (Da)
0 h in PVA1799 medium	16.113	112,981
2 h in PVA1799 medium	16.431	84,803
0 h in PVA124 medium	16.667	73,762
72 h in PVA124 medium	16.767	66,544

with polymerization of 1700 from initial average molecular weight 112,981 to 98,827 Da and 84,803 Da, respectively. For PVA124, less PVA124 with polymerization of 400 was degraded by two these strains within 72 h. Residual PVA in PVA1799 media was precipitated by acetone and washed. The residual PVA was used to prepare PVA media, and strains Nos. 1 and 7 could not grow on these media again. This indicated that strains Nos. 1 and 7 could not further utilize these residual PVA, and the extracellular PVA-degrading enzymes secreted by strains Nos. 1 and 7 were selective to their substrates. The reason of such selectivity of PVA-degrading enzymes was probably that polymer could not link steadily with degrading enzyme when its degree of polymerization was less than certain amount and then the degradation process stopped [10]. PVA was not detected in cell-free extract of these two strains.

Strains Nos. 2–6 were also individually cultivated in PVA1799 and PVA124 media, respectively. Table 4 shows that strains Nos. 2 and 6 could utilize PVA1799; and strain No. 4 could grow in both PVA1799 and PVA124 media. Strain No. 3 utilized neither PVA1799 nor PVA124. The strains that could not produce extracellular PVA-degrading enzymes degraded PVA by membrane-oriented or intracellular PVA-degrading enzymes [11]. Thus, these membrane-oriented or intracellular PVA-degrading enzymes were also selective to their substrates. Strain No. 3 was possibly a symbiotic bacterium and its growth depended on the degrading products of PVA.

Table 4
Cell growth of strain Nos. 2–6 growing on PVA1799 and PVA124 media

	Strain no.				
	2	3	4	5	6
OD_{600}	0.05[a]	0.01[a]	0.21[a]	0.03[a]	0.06[a]
	0.14[b]	0.02[b]	0.15[b]	0.09[b]	0.18[b]

[a] Cultured in PVA1799 medium.
[b] Cultured in PVA124 medium.

The molecular weight distribution of PVA detected within cells of mixed culture was less than 10,000 Da (Table 5). Such molecular weight distribution peaks disappeared after cells were transferred to basic medium without PVA and cultivated for 2 days. This suggested that some microorganisms in the mixed culture produce intracellular PVA-degrading enzymes for the further utilization of PVA-degrading intermediates.

3.4. T-RFLP results

By cultivating the mixed culture in basic medium, 1 g l^{-1} of PVA1799 was degraded completely within 4 days. The abundance of the mixed culture in PVA degradation course was analyzed through T-RFLP method. The 16S rDNA of the mixed culture was cut by *Alu*I, *Hha*I and *Hae*III. The results are shown in Fig. 7. Table 6 gives the possible microorganisms and their relative abundances during the degradation. The relative abundances of *Pseudomonas* sp., *Flavobacterium* sp., *Streptococcus* sp. and *Micrococcus* sp. increased with time. *Pseudomonas* sp., *Flavobacterium* sp. and *Micrococcus* sp. were dominant microorganisms in the mixed culture at the end of cultivation and their relative abundances reached 30.6, 17.2 and 19.7%, respectively. Especially, *Micrococcus* sp. increased from 0.7% at 64 h to 19.7% at 96 h. The relative abundance of *Bacillus* sp.

Table 5
Molecular weight distribution of PVA in cells

Name	Migration time (min)	Molecular weight (Da)
Peak 1	19.119	9536
Peak 2	21.132	975

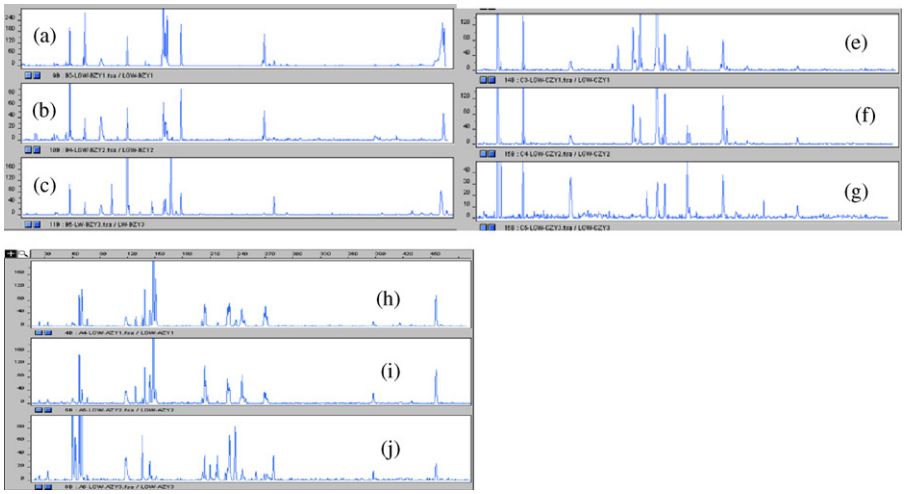

Fig. 7. T-RFLP results of the mixed culture. (a–c) 16S rDNA treated by *Hha*I at 32, 64 and 96 h, respectively; (e–g) 16S rDNA treated by *Hae*III at 32, 64 and 96 h, respectively; (h–j) 16S rDNA treated by *Alu*I at 32, 64 and 96 h, respectively.

was 19.5% at the beginning of the degradation, then decreased with time. *Rhodococcus* sp. and *Sphingomonas* sp. increased from 4.9% at the beginning to 11.1% at 64 h, but decreased to only 1.2% in the mixed culture at the end of cultivation. *Paenibacillus* sp. also increased from 24.2% at the beginning to 25.4% at 64 h, and then decreased to 2.0%. The relative abundances of unmatched microorganisms were 15.3, 11.6 and 11.8% at 32, 64 and 96 h, respectively.

Thus, the microorganisms in the mixed culture degraded PVA1799 through a synergic effect. When cells were removed by centrifugation, the capability of PVA degradation of the supernatant decreased more than 70%. So, beside enzymes secreted by cells in this mixed culture to fermentation broth, the cells contributed a lot to the excellent PVA degradation capability of the mixed culture. The strains that could not produce extracellular PVA-degrading enzymes degraded PVA by membrane-oriented or intracellular PVA-degrading enzymes [11]. And PVA fragment in low molecular weigh was found to enter cells and be consumed further. Then the PVA degradation model of this mixed culture was supposed. At the beginning of cultivation, some microorganisms that could cut PVA1799 grew up firstly; extracellular and membrane-oriented PVA-degrading enzymes produced by these microorganisms cut PVA1799 to smaller molecules. With the degree of polymerization of PVA decreasing, the number of those microorganisms which could utilize such smaller PVA fragments increased. At the same time smaller PVA fragments could enter cells of some microorganisms, which produced intracellular PVA-degrading enzymes for the further degradation of the intermediates.

The purification of PVA-degrading enzymes in the mixed culture and the study on biodegradation mechanism of PVA by the mixed culture are being carried out.

Table 6
T-RFLP of the mixed culture during degradation of PVA1799

Strains	Relative abundance of the strain in the mixed culture (%)		
	32[a]	64[a]	96[a]
Pseudomonas sp.	2.7	6.2	30.6
Sphingomonas sp.	4.9	11.1	1.2
Rhodococcus sp.	7.0	5.5	4.0
Bacillus sp.	19.5	12.8	1.8
Brevibacterium sp.	6.4	7.6	5.8
Flavobacterium sp.	11.6	13.3	17.2
Micrococcus sp.	N	0.7	19.7
Streptococcus sp.	N	0.8	3.6
Leptothrix sp.	8.4	5.0	2.3
Paenibacillus sp.	24.2	25.4	2.0
Unmatched	15.3	11.6	11.8

N: not detected.
[a] Culture time (h).

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