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Biodegradation of Poly(vinyl alcohol) with High Isotacticity

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ABSTRACT: A bacterium having biodegrading activity with poly(vinyl alcohol) (PVA) was isolated from an activated sludge sample of a textile dyeing factory which deals with PVA. The bacterium was identified as a strain of *Pseudomonas* sp. and designated strain A-41. The results of the degradation of commercial PVA having atacticity altered triad-isotacticity from 21% to 13%. This suggests that the isotactic sequences in PVA are susceptible to biodegradation preferentially. PVA with high isotacticity was found to be easily degraded in comparison with commercial PVA.

KEY WORDS Poly(vinyl alcohol) / Stereoregularity / Biodegradation / *Pseudomonas* / Isotacticity / Chain Scission /

Suzuki *et al.* investigated the biodegradation of poly(vinyl alcohol) (PVA) using a bacterium, *Pseudomonas* O-3 and this bacterium was used for treating wastewater which contains PVA.[1,2] Sakazawa *et al.* confirmed the presence of the symbiotic bacteria for PVA.[3] Sakai *et al.* proposed a degradation mechanism by a suitable model using various alcohols with low molecular weights as substrates, and they clarified that the chain scissions of PVA occur at skeletal C-C linkages by the successive oxidation of hydroxyl groups followed by hydrolysis.[4-6]

However, detailed reports on the influence of the stereoregularity of hydroxyl groups in PVA on the biodegradation have not been published to date. Studies on the preparation of high molecular weight PVA with high syndiotacticity, which is derived from a photo-emulsion radical polymerization of vinyl pivalate at low temperature, have been made at our laboratory.[7] PVA obtained by this process showed excellent physical properties owing to its high stereoregularity.[8]

The present report describes the isolation and characterization of a new PVA-degrading bacterium, and changes in tacticity of atactic- and isotactic-PVA with degradation using the bacterium.

EXPERIMENTAL

PVA Samples

The degrees of polymerization ($\overline{P_n}$), contents of 1, 2-glycol linkage and triad-tacticities of used PVA samples are shown in Table I. PVA (PVA-I) was a product of Kuraray Co., Ltd. and had a degree of saponification of not less than 99.9%. It was purified by repeated reprecipitation of the aqueous solution with methanol, and dried *in vacuo*. PVA having high isotacticity (PVA-II) was prepared by cationic polymerization of *t*-butyl vinyl ether with boron trifluoride etherate at -78°C followed

Table I. Properties of the PVA samples

Sample	$\overline{P_n}$	Content of 1,2-glycol linkage/mol%[a]	Triad-tacticities/%[b]		
			I	H	S
PVA-I	1750	1.6	21	50	29
PVA-II	4500	<0.1	72	24	4

[a,b] Determined by ^{1}H NMR analysis.
[b] I, isotacticity; H, heterotacticity; S, syndiotacticity.

Table II. Compositions of culture media

Components	PVA-Y/g l^{-1}	PVA-TY/g l^{-1}
PVA[a]	1.0	1.0
Yeast-extract	1.0×10^{-2}	4.0
Tryptone	—	2.0×10
KH_2PO_4	1.6	1.6
K_2HPO_4	2.0×10^{-1}	2.0×10^{-1}
NH_4NO_3	1.0×10^{-1}	1.0×10^{-1}
$FeSO_4\cdot7H_2O$	2.0×10^{-2}	2.0×10^{-2}
$CaCl_2\cdot2H_2O$	6.4×10^{-2}	6.4×10^{-2}
$MgSO_4\cdot7H_2O$	1.0×10^{-1}	1.0×10^{-1}
NaCl	2.0×10^{-2}	2.0×10^{-2}

[a] PVA-I was used in usual incubation.

by cleavage of the ether linkage of this polymer using hydrogen bromide at 0°C.[9] This PVA had residual ether groups at less than 0.1 mol%.

Culture Media

The compositions of culture media are listed in Table II. Yeast-extract (Difco Lab.) and several inorganic salts were added to distilled water and pH was adjusted to 7.2. An aqueous solution of PVA (10 g l^{-1}) was further added to give a final concentration of 1 g l^{-1}. The medium (PVA-Y medium) was sterilized at 120°C for 20 min in an autoclave and used for the isolation of PVA-degrading bacteria. To improve bacterial growth, tryptone (Difco Lab.) was added to the PVA-Y medium designated the PVA-TY medium. For solid media, agar was added at 15 g l^{-1}.

Isolation and Purification of Bacterium

Activated sludge samples were obtained from a wastewater treatment facility in a textile dyeing factory (Nishiwaki-city, Hyogo Pref.) which deals with PVA, and used as a bacterial source. The samples were diluted with distilled water and plated on PVA-Y medium. Colonies on the plates were tested for PVA-degrading activity after 10 days of incubation at 30°C. Activity was detected as clear halos around the colonies on the plates when a mix solution (1 : 1) of KI–I_2 solution and 4% aqueous solution of boric acid was poured onto the plates. The halo formation was due to loss of the iodine–PVA coloration reaction by PVA degradation. The colonies with the activity were streaked onto the plates and incubated at 30°C. These operations were repeated several times for purification of bacteria.

Identification of Bacterium

The identification of isolated bacterium was performed on the basis of the classification of Bergey's Manual.[10]

PVA Degradation Test

After pre-incubation in PVA-TY medium at 30°C for 7 days, the cells were collected by refrigerated centrifuging at 12,000 rpm for 10 min. The cells were washed three times with 10 mM phosphate buffer solution and suspended in the same solution. A given amount of cell suspension was inoculated into 100 ml of PVA-Y medium in a 300 ml Erlenmeyer flask. Incubation for the degradation test was carried out at 30°C on a shaker at 150 rpm.

Biomass concentration in a culture was estimated by optical density at 660 nm (O.D.$_{660}$) with a Hitachi U-2000 spectrophotometer.

A supernatant solution of a culture was prepared by refrigerated centrifuging at 12,000 rpm for 10 min. One milliliter of the supernatant solution was diluted with distilled water to 25 ml, followed by adding 15 ml of 4% aqueous solution of boric acid. Three milliliters of KI–I_2 solution were then added to the solution, and the solution was diluted with

distilled water to 50 ml. After the solution was maintained at 25°C for 30 min, absorbance at 690 nm was measured. The concentration of PVA in a culture was calculated from absorbance.

Total organic carbon concentration (TOC) of the supernatant solution was measured by Shimadzu model TOC-500. Relative viscosity of the supernatant solution to water at 30°C was determined by viscosity measurement using an Ostwald-viscometer.

Residual PVA in a culture was recovered by dialyzing the supernatant solution for 7 days against water. The dialyzed solution was condensed and poured into MeOH. The deposits were washed with MeOH and dried *in vacuo* at 60°C. ^{1}H NMR spectroscopy was carried out with a JEOL JNM EX-270 using 1% dimethyl sulfoxide-d_6 solution of PVA.

RESULTS AND DISCUSSION

Isolation and Characterization of PVA-Degrading Bacteria

PVA-degrading bacteria were screened from activated sludge samples collected from the wastewater treatment facility at a textile dyeing factory. Clear zones were visually observed around six colonies on the PVA-Y medium.

Microscopic observation revealed that each colony consisted of more than two kinds of bacteria. After repeated subculturing, only one isolate with PVA-degrading activity was obtained. By scanning electron microscopy, the purity of the isolate was verified.

The bacterium was gram-negative, catalase- and oxidase-positive, aerobic and rod-shaped. Its mobility was also observed. These characteristics of the bacterium allowed tentative assignment of the genus *Pseudomonas*. This bacterium was designated *Pseudomonas* sp. strain A-41.

Strain A-41 grew slowly, degrading PVA on PVA-Y agar plates and it required about one week to form a 1 mm-diameter colony. However, it could not utilize PVA without a yeast-extract. This shows that it requires supplemental nutrients such as animo acids and/or vitamins. Hashimoto *et al.* reported that the PVA-degrading bacterium, *Pseudomonas vesicularis* var. *povalolyticus* PH, required a yeast-extract or thiamine and three amino acids to degrade PVA.[11] Sakazawa *et al.* also reported that *Pseudomonas* sp. VM15C required pyrroloquinoline quinone, produced by the symbiotic *Pseudomonas putida* VM15A, and in addition, required a yeast-extract to degrade PVA.[3,12] Therefore, strain A-41 seems to have similar nutritional requirements to these two PVA-degrading *Pseudomonas*. Its nutritional requirements are still unknown.

Degradation of Commercial PVA

Strain A-41 was incubated at 30°C in PVA-Y medium containing 1 g l^{-1} PVA-I. The amount of PVA in the culture measured by iodometry decreased with increase of incubation time and approached approximately half the initial amounts in 10 days.

Residual PVA in the culture after 10 days of

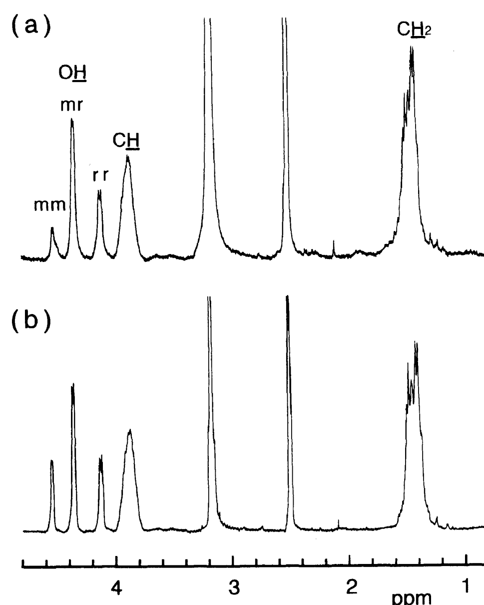

Figure 1. ^{1}H NMR spectrum of PVA after biodegradation; (a), sample; (b), control.

incubation was recovered. Figure 1a shows the ^{1}H NMR spectrum of PVA recovered. Three resonances occurred in the hydroxyl proton regions and were assigned to isotactic (*mm*), heterotactic (*mr*), and syndiotactic (*rr*) sequences. Figure 1b shows the spectrum of PVA recovered from a control medium without bacterial inoculation. There were differences in peak intensities of hydroxyl protons in the two spectra. The tactic contents of *mm*, *mr*, *rr* calculated from their peak areas for PVA recovered from the control medium were 21%, 50%, and 29% respectively, whereas those of PVA after degradation, 13%, 55%, and 32% respectively. No marked differences were observed between the spectrum of PVA recovered from the control medium and that of the original PVA. Therefore, the decrease in the content of the isotactic sequence was considered due to biodegradation.

Degradation of Isotactic PVA

Degradation test of PVA-II was made using strain A-41. Incubation was carried out at 30°C in PVA-Y medium containing PVA-II at 0.6 g l^{-1}. For comparison of degradation characteristics, incubation was simultaneously done in PVA-Y medium containing PVA-I at the same polymer- and bacterium-concentration as in the case of the PVA-II culture. Figure 2 shows the changes of residual PVA in the culture with incubation time by iodometry. The amounts of PVA-I in the culture decrease to half the initial amounts in 10 days, whereas those of PVA-II in the culture decrease to 20% of the initial amounts in 4 days, and no PVA-II was detected at all by iodometry after 8 days.

Figure 3 shows changes in relative viscosities of culture filtrates with incubation time. The viscosity of PVA-I culture gradually lowered, whereas that of PVA-II culture rapidly dropped.

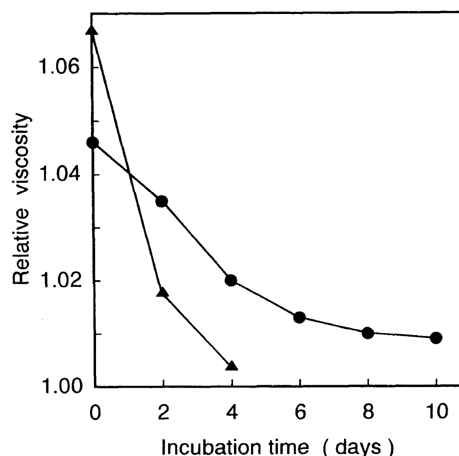

Figure 3. Changes in relative viscosities of culture filtrates with incubation time; ●, PVA-I; ▲, PVA-II.

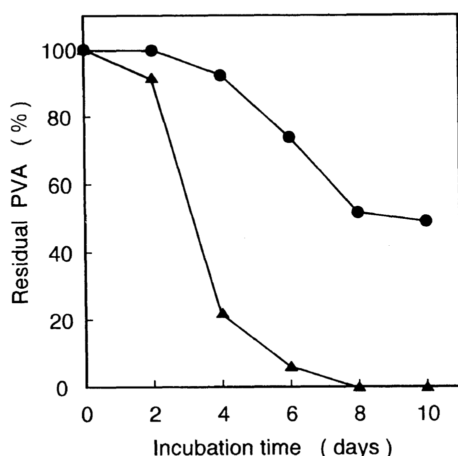

Figure 2. Changes in percentages of residual PVA in media with incubation time; ●, PVA-I; ▲, PVA-II.

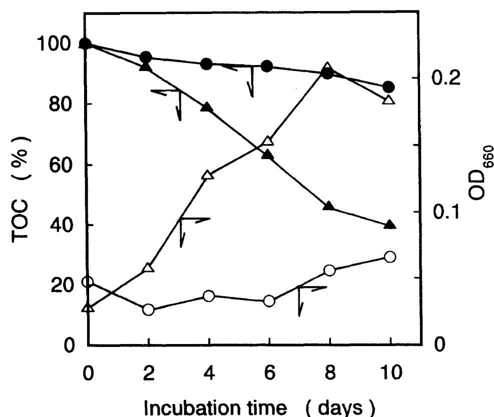

Figure 4. Changes in TOC and OD_{660} of culture media with incubation time; ○, ●, PVA-I; △, ▲, PVA-II.

Figure 4 shows plots of TOC and biomass concentration in each culture against incubation time. The decrease of TOC in PVA-II culture is larger than that in the PVA-I culture. For 10 days of incubation, 15% of initial amounts of PVA-I was metabolized, whereas 60% in PVA-II. In the PVA-II culture, the bacterium easily grew compared with that in PVA-I. PVA-II was subjected to assimilation and it promoted proliferation of the bacterium.

PVA-II undergoes decomposition fairly to PVA with low molecular weight faster than PVA-I. PVA-II seems to undergo easy chain scission by biodegradation. There are some differences in $\bar{P}_n$ and content of 1, 2-glycol linkage between PVA-I and PVA-II. It has been reported by Suzuki *et al.* that the degrees of polymerization and saponification should have no influence on the biodegradation of PVA.[1] $\bar{P}_n$ of PVA-II is higher than that of PVA-I and the content of 1, 2-glycol linkage in PVA-II is significantly lower than that in PVA-I. These differences cannot be assumed to be responsible for biodegrading activity.

PVA with high isotactic content was subject to biodegradation and isotactic content in commercial PVA was greatly reduced by biodegradation. This suggests that isotactic sequences are severed preferentially by enzymatic reaction. Chain scissions in PVA-II proceeded at a number of sites in the chain skeleton to produce small molecules of short chain length. This resulted in an easy assimilation. If the chain scissions of isotactic sequences in PVA-I will proceed, the chain lengths of molecules yielded by scission will certainly become longer than those in PVA-II. The ratios of syndiotactic sequences in degraded PVA should thus increase and consequently, further degradation reactions will not occur easily.

Sakai *et al.* propose that hydroxyl groups in PVA are oxidized to form ketones by secondary alcohol oxidase (SAO) and the resulting ketones lead to β-diketones by subsequent oxidation reactions.[6] Chain scission must occur, by this view by Sakai, at sites of β-diketones by a function of β-diketone hydrolase.

The infrared spectrum of residual PVA-I recovered after degradation clearly exhibited a new peak in the vicinity of 1700 cm^{-1} assigned to C=O absorption. This suggests that the biodegradation of PVA using the strain A-41 proceeds by following the consideration proposed by Sakai *et al.*[6]

According to this degradation process, differences in degrading activities may arise in the oxidation steps of hydroxyl groups. Differences in tacticities disappear when the diketone is formed from two adjacent hydroxyl groups owing to oxidation reaction. Effects of tacticity in PVA on biodegrading activity seem attributable to the stereo structure of oxidase and its functionality, and ease of access of oxidase to the active site of the polymer.

SAO showed the same decomposing activity with 3-hexanol or 4-heptanol as with PVA.[4] The activity of SAO was also observed with 3,4-nonanediol, but 2,4-pentadiol. Sakai *et al.* showed that in order to act on a substrate, the oxidase required a sequence joining more than three carbon atoms to both sides of the α-carbon attached to a hydroxyl group. The oxidase used must exhibit the stereoselectivity for oxidation reaction, since the substrate was required to have relatively long sequence for molecular recognition.

CONCLUSIONS

The data in this work support the fact that the stereoregularity of PVA affects remarkably its biodegrading reaction and PVA having high isotacticity undergoes easily a biodegradation more than commercial PVA.

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