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A modified method for isolating poly(vinyl alcohol)-degrading bacteria and study of their degradation patterns

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Abstract

An addition of catalase or peroxidase into an agar plate containing poly(vinyl alcohol) (PVA), was effective for the isolation of PVA-degrading microorganisms. A Gram-negative bacterium, strain TK-2 (γ-group of proteobacteria), rapidly degraded a high molecular weight PVA to low molecular weight material after 1 day thereby producing oligomers of PVA as shown by gel permeation chromatography. Conversely, *Sphingomonas* strain TJ-7 did not produce any PVA oligomers, suggesting that the strain TJ-7 degraded PVA from the terminal ends of the molecules, whereas the strain TK-2 cleaved PVA at random.

Introduction

Poly(vinyl alcohol) (PVA), a water soluble polymer prepared by the hydrolysis of poly(vinyl acetate), is widely used in industrial applications, such as adhesives and as sizing agent in textiles. It has also been used for cell immobilization (Pattanapipitpaisal *et al.* 2001). Recently, PVA has attracted attention as the biodegradable backbone in sugar and fatty acid branched polymers (Tokiwa *et al.* 2000). PVA is produced in greater quantities than any other water-soluble synthetic polymer. The world production of PVA is about 650 000 tons per year and the large amount of used and discarded PVA has become a significant waste problem.

The distribution and number of microorganisms able to degrade PVA are limited in comparison with those that can degrade aliphatic polyesters, e.g., poly(hydroxyalkanoate) (Nishida & Tokiwa 1993) and poly(ε-caprolactone) (Pranamuda *et al.* 1995). Suzuki *et al.* (1973) were the first to isolate a PVA-degrading bacterium, *Pseudomonas* O-3. In later studies, only a restricted number of microorganisms, such as *Pseudomonas* spp. (Watanabe *et al.* 1976, Sakai *et al.* 1998), *Alcaligenes faecalis* (Matsumura *et al.* 1994), and *Bacillus megaterium* (Mori *et al.* 1996), have been reported to degrade PVA. So far, several reports on PVA degradation have dealt with symbionts and the difficulty of isolating PVA-degrading microorganisms has been pointed out in earlier reports (Sakazawa *et al.* 1981, Shimao *et al.* 1982, 1986). One reason may be the difficulty of isolating a single strain by conventional plate culture methods. This vagueness led us to seek a technique for isolation of individual PVA-degrading microorganisms.

The sparsity of information available regarding microbial degradation of PVA, despite the large amounts consumed, has made study of its degradation necessary. In this report, a modified method for isolation of PVA-degrading bacteria and the role of their PVA-degradabilities are demonstrated. Degradation

patterns of PVA by the isolated strains are also discussed.

Materials and methods

Materials

PVA (degree of polymerization, DP, 1700; degree of saponification, 99%) was received as a gift from Kuraray Co., Ltd. (Osaka, Japan). Pyrroloquinoline (PQQ), a coenzyme of PVA-dehydrogenase, was purchased from Wako Pure Chemical Industries (Osaka, Japan). The enzymes used were horse radish peroxidase (Toyobo Co. Ltd. Osaka, Japan) and bovine liver catalase (Wako Pure Chemical Industries, Osaka, Japan).

Isolation of PVA-degrading bacteria by a modified method

The microorganism consortia capable of degrading PVA were obtained by repeatedly enriching the medium and then spreading on PVA agar medium. The PVA agar medium consisted of 5 g PVA, 100 mg yeast extract and 40 μg PQQ in 1 l of inorganic salts basal medium (0.5 mg $Na_2Mo_4 \cdot 2H_2O$, 0.5 mg $MnSO_4$, 10 mg NaCl, 1000 mg $(NH_4)_2HPO_4$, 1600 mg K_2HPO_4, 0.5 mg $Na_2WO_4 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 200 mg $MgSO_4 \cdot 7H_2O$, 200 mg KH_2PO_4 and 20 mg $CaCl_2 \cdot 2H_2O$), pH 7.5. Before spreading the enrichment culture on the PVA agar plates, each plate was previously overlaid with 5 units of catalase or 0.3 units of peroxidase. The plate cultures were incubated at 30 °C.

Degradation of PVA by the isolated strains

The degradation of PVA by the isolated strains was carried out aerobically (180 r.p.m.) at 30 °C with 0.5 g PVA in a 500 ml Erlenmeyer flask containing 100 ml basal medium of inorganic salts plus 40 μg PQQ l^{-1}. Residual PVA in the culture broth was measured by iodometry, based on the green color produced by the reaction of PVA with iodine in the presence of boric acid, according to the method of Finley (1961). Before measuring the residual PVA, cell growth was followed by the turbidity of the culture broth at 660 nm and dry weight of cells was estimated from the calibration curve ($OD_{660} = 0.3$ mg dry cell wt ml^{-1}) after drying at 105 °C for 16 h. Water-soluble total organic carbon (TOC) concentration in the culture broth after filtration with a 0.22 μm filter membrane (Millipore Coporation, USA) was measured with a TOC-5000 analyzer (Shimadzu Co. Ltd.). The polymers present in the culture broth before and after the degradation test were directly determined by gel permeation chromatography with a refraction index detector (GPC, HLC-8120 TOSOH, Tokyo, Japan).

PVA degradation by the resting cells and the culture filtrate

The cell suspension was harvested from the PVA culture broth by centrifugation at 4500 $\times$ *g* for 20 min at 4 °C, washed twice with 0.01 M phosphate buffer (pH 7), followed by resuspension of the cells in the same buffer. The cell suspension was employed as resting cells. The culture broth, filtered through a filter membrane (pore size, 0.22 μm) and the resting cells were used for PVA degradation in the reaction mixture. The mixture was then reacted following the method of Suzuki *et al.* (1973). H_2O_2 formation was followed spectrophotometrically at 410 nm according to the procedure based on the titanium reaction for H_2O_2 (Teranishi *et al.* 1974).

Results and discussion

Isolation of PVA-degrading bacteria by a modified method

After repeated enrichment culturing with PVA liquid medium, the microorganisms in the PVA-degrading consortia grew on PVA agar plates with different colony appearances. However, none of the colonies showed PVA-degradability in the liquid culture even when the culture medium was supplemented with PQQ.

Two enzymes involved in PVA degradation are PVA oxidase, an extracellular enzyme, and PQQ-dependent PVA dehydrogenase, an intracellular enzyme (Shimao *et al.* 1982, 1986). An aldolase-type PVA degradation mechanism involving the apoenzyme of PVA dehydrogenase has also been reported (Matsumura *et al.* 1999). The reaction by PVA-oxidase is accompanied by generation of H_2O_2. Since H_2O_2 is toxic, it can be presumed to inhibit colony formation of PVA-degrading bacteria.

Interestingly, a number of colonies were formed when catalase was added to the PVA agar plate (Figure 1). In addition, no colony was observed on the agar plate added with equal amount of inactivated catalase

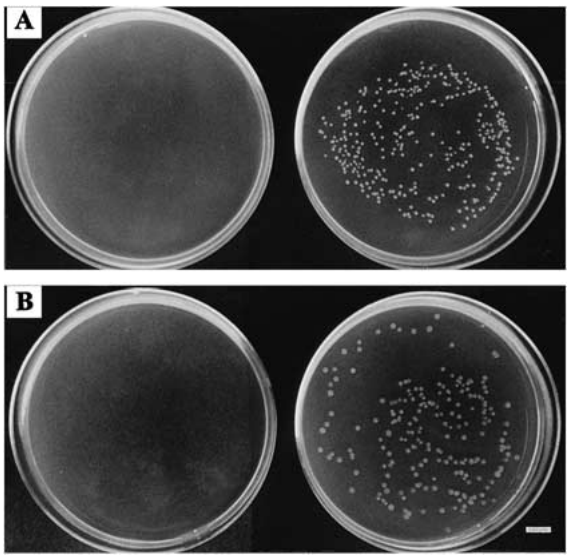

Fig. 1. Appearance of the PVA-degrading bacteria colonies on PVA agar plates by addition of catalase (right) compared to addition of inactive catalase (left) when plated out from the enrichment medium after 24 h incubation; (A) strain TK-2 and (B) strain TJ-7. Bar scale = 1 cm.

(boiling for 10 min). The minimum amount of catalase for the formation of PVA-degrading bacteria colonies was ca. 1 unit (0.016 unit cm^{-2} of agar plate).

To confirm the inhibition of colony formation by H_2O_2, a similar experiment using peroxidase was also carried out. The addition of peroxidase, which exhibited H_2O_2 degradation similar to catalase, enabled the PVA-degrading bacteria to form colonies. No colony was observed on PVA agar medium with an inactive peroxidase. From these results, it seems reasonable to assume that when PVA-degrading bacteria degrade PVA, they generate H_2O_2 to a degree that inhibits their own growth. However, in liquid culture, these strains grew well even with no addition of catalase in the culture media. This is probably because the H_2O_2 generated into the medium was not present in the vicinity of the bacteria. Addition of catalase and peroxidase, which decompose H_2O_2, was considered to be an appropriate method for isolating PVA-degrading bacteria. We readily isolated two PVA-degrading bacteria, strains TK-2 and TJ-7, for further study.

The two isolated strains are both Gram-negative rods. Strain TK-2 forms a circular white colony, whereas strain TJ-7 forms a circular yellow colony. Taxonomic studies and the results of analysis based on 16S rRNA gene sequences (ca. 1400 nucleotides) revealed that strain TK-2 belongs to γ-subclass of proteobacteria but is not closely related to any known

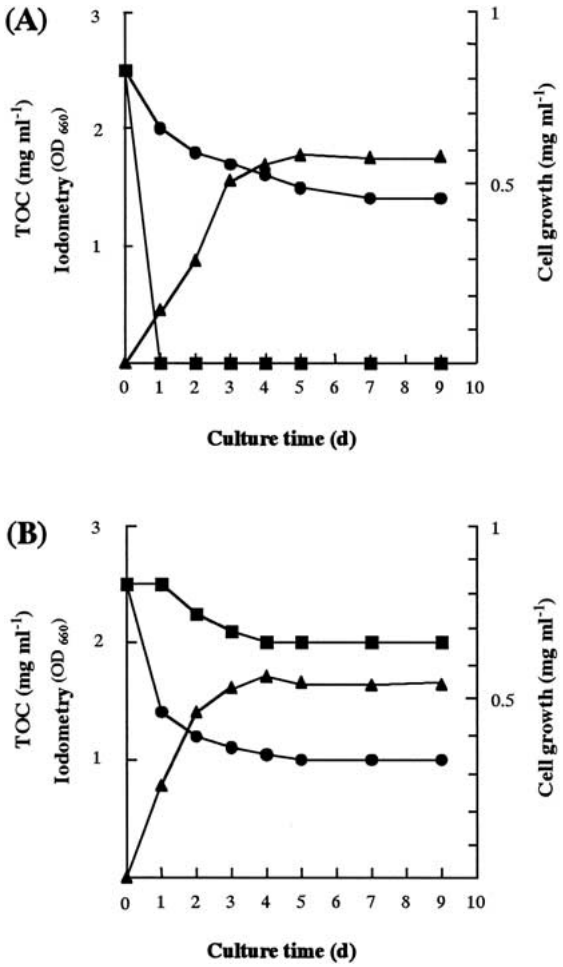

Fig. 2. Time course of poly(vinyl alcohol) degradation by (A) the isolated strain TK-2 and (B) the isolated strain TJ-7 at 30 °C: cell growth (▲), iodometry (■) and TOC, water-soluble total organic carbon (●).

genera, with only 88% similarity to *Nitrosococcus* sp. Strain TJ -7 was classified as *Sphingomonas* sp. with 96% similarity.

Degradation patterns of PVA by the isolated strains

Degradation patterns of PVA-degrading bacteria strain TK-2 and TJ-7 were studied in the liquid culture medium with PVA as a carbon source. The degree of PVA molecule fragmentation was determined by iodometry. The PVA molecule fragments with molecular weight of less than 2000 had no iodometry reaction. Accumulation of water-soluble organic carbon containing PVA and degraded PVA substances in the culture medium was estimated as TOC value (PVA contains 54% organic carbon in the molecule). Fig-

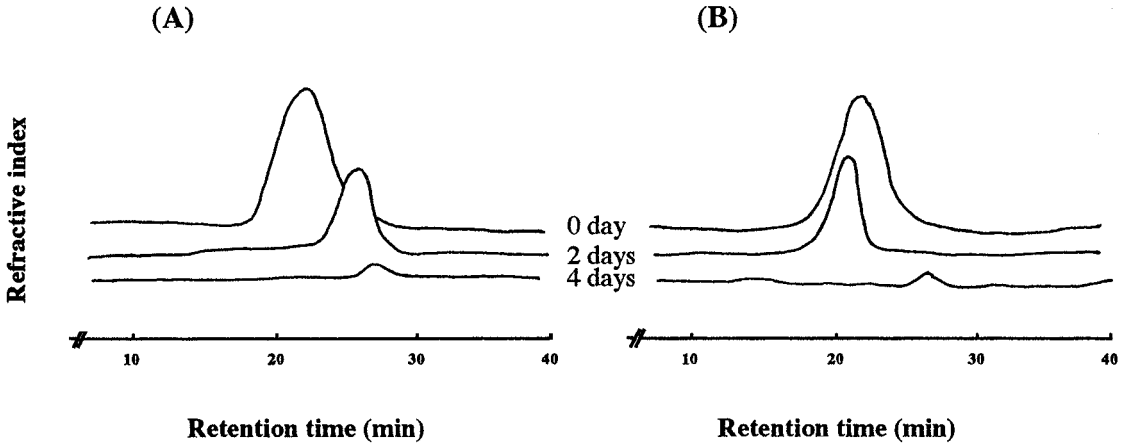

Fig. 3. Gel permeation chromatograms of poly(vinyl alcohol) after degradation by (A) the isolated strain TK-2 and (B) the isolated strain TJ-7 in liquid culture at 30 °C.

ure 2A shows typical time course of PVA degradation by strain TK-2 which had strong PVA fragmentation capability but the final TOC concentration in culture broth only decreased gradually after 1 day of cultivation. GPC was used to analyze the change of PVA molecular weight distribution in the process of degradation. The main peak was shifted relative to that of control, and oligomer peak of PVA was detected after 2 days of cultivation. This confirmed that strain TK-2 randomly fragmented PVA (Figure 3A).

On the other hand, strain TJ-7 grew rapidly and had TOC reduction capability higher than strain TK-2. However, iodometry showed that strain TJ-7 had very weak PVA fragmentation capability (Figure 2B). GPC patterns of PVA in culture medium of TJ-7 were analyzed after 2 days of cultivation. The peak of the original PVA was detected but no oligomer peak of PVA was observed (Figure 3B). This indicates that strain TK-2 randomly fragmented PVA, while strain TJ-7 degraded PVA from the ends of the molecules. After 4 days, the main chains of PVA were completely degraded and a small amount of degraded PVA substances was detected in the culture broth (Figures 3A and 3B). A significant increase in the cell growth was observed, indicating that the isolated strains were able to assimilate the degraded PVA substances.

To verify the difference in degradation patterns of the two isolated strains, PVA fragmentation and H_2O_2 formation by the resting cells (washed cells) and the culture filtrate in the enzymatic reaction mixture were examined. As shown in Table 1, PVA degradation and H_2O_2 formation by PVA oxidase (extracellular enzyme) in the culture filtrate of strain TK-2 was detected. Some degree of PVA fragmentation was also observed in the resting cells of this strain. In contrast, no PVA fragments and H_2O_2 formation caused by PVA oxidase were observed in the culture filtrate of strain TJ-7 (data not shown). Strain TJ-7 produced only PVA dehydrogenase which has binding affinity at the terminal end of PVA chains.

Table 1. Poly(vinyl alcohol) fragmentation and hydrogen peroxide (H_2O_2) formation by the fractions obtained from isolated strain.

Isolated strain	Fraction	Degree of PVA fragmentation[a] (%)	H_2O_2 formation (μmol min^{-1})
TK-2	Resting cells	19	0
	Culture filtrate	76	1.98

[a]Degree of PVA fragmentation was calculated from the % decrease of the initial green color (iodometry) in the reaction mixtures. The reaction mixture contained 5 ml resting cell suspension or culture filtrate and 5 ml of 2% (w/v) PVA in 0.2 M phosphate buffer, pH 7. The mixture was incubated on a rotary shaker at 100 rpm and 30 °C for 12 h.

We found that strain TJ-7 had self-inhibition of colony formation by H_2O_2 when it was cultured on glucose agar medium without an addition of catalase. This made insufficient our earlier hypothesis that the difficulty in isolation of PVA-degrading microorganisms is only due to the presence of H_2O_2-generated by PVA oxidase. The most reasonable explanation is probably that PVA-degrading bacteria generate H_2O_2 not only by the reaction of PVA oxidase but also by their own metabolic reaction. The sensitivity to H_2O_2 is probably one of the reasons why PVA-degrading microorganisms are rarely isolated.

Our method of using an agar medium added with catalase or peroxidase is an effective method for isolating PVA-degrading bacteria, which do not form colonies when conventional methods are used. By using our method, new PVA-degrading strains having different PVA cleavage-type were isolated and reported. Since waste water from textile manufacturing after desizing process contains large amounts of PVA, these isolated strains can be used as the seeds to accelerate the rate of PVA degradation in the biological waste water treatment systems.

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