

Symbiotic Degradation of Polyvinyl Alcohol by *Novosphingobium* sp. and *Xanthobacter flavus*.

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Polyvinyl alcohol (PVA) must be removed from the waste water before discharge. In this study, we isolated some bacteria from the activated sludge. One of them, *Novosphingobium* sp. strain P7, could degrade PVA in the minimal medium supplied with methionine. We also isolated *Xanthobacter flavus* strain B2, which did not have PVA-degrading activity. Co-culture of strain P7 and B2 could degrade PVA without supplying the methionine. It seems likely that strain B2 supplied methionine for the growth of strain P7, which led to the degradation of PVA.

Key words: Polyvinyl alcohol, Biodegradation, Symbiosis, *Novosphingobium* sp., *Xanthobacter flavus*

Polyvinyl alcohol (PVA), a water soluble polymer prepared by the hydrolysis of polyvinyl acetate, is widely used in industrial applications, such as adhesives and sizing agent in textiles¹⁰. 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. PVA is well-known biodegradable polymer but not easily degraded in the environment. Therefore, the large amount of used and discarded PVA has become a significant waste problem. In previous study, several microorganisms, which can degrade aliphatic polyesters like as poly (hydroxyalkanoate) and poly (ϵ -caprolactone), are reported^{2,10,13}. In case of PVA, some microorganisms, such as *Pseudomonas* O-3¹², *Pseudomonas vesicularis* PD⁷, *Alcaligenes faecalis*⁵, *Bacillus megaterium*⁶, and *Sphingopyxis* sp. PVA3¹⁴, have been reported as PVA-degrading bacteria. Furthermore, several reports on PVA degradation have dealt with symbiont and the difficulty of isolating PVA-degrading microorganisms has been pointed out in earlier reports^{1,3,8,11}. 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 little information available regarding microbial degradation of PVA, despite the large amounts consumed, has made study of its degradation necessary. In the paper, we present the study on the degradation behavior of PVA by an acclimated PVA-degrading microorganism, and responsible

heterogeneous microbial culture involved. Degradation patterns of PVA by the isolated strains are also discussed.

Activated sludge samples were obtained from the wastewater treatment plant. For isolation of PVA-degrading bacteria from the activated sludge, PVA minimal medium, which contained KH_2PO_4 (0.4 g/l), K_2HPO_4 (3.2 g/l), $(\text{NH}_4)_2\text{SO}_4$ (1 g/l), MgSO_4 (1 g/l), NaCl (0.1 g/l), FeSO_4 (10 mg/l), yeast extract (0.05 g/l), and the various concentration of PVA (Wako Pure Chemicals, Osaka; degree of polymerization degree: 500), was used. Activated sludge was inoculated into the 0.6% PVA medium and incubated at 30°C with shaking. The concentration of residual PVA was measured by the previously described method with slight modification¹. Briefly, the culture supernatant was diluted to 0.3 ml with distilled water, and then 0.45 ml of 4% boric acid and 90 μl of I_2 -KI solution were added. Then, the mixture was diluted to a volume of 1.5 ml with distilled water and analyzed at 660 nm to estimate PVA concentration. Cell growth was measured the optical density at 600 nm (OD600). After 6 days of incubation, increasing of cell growth and degradation of PVA were appeared, and almost of all PVA was degraded after 10 days (Fig. 1). To isolate PVA-degrading bacteria, bacterial cells in the culture were collected by centrifugation, resuspended in the distilled water, and spread on the LB agar plates⁹. After a week of incubation, several colonies were formed. Formed colonies were picked up and grown in the LB liquid medium. Grown

cells were collected, washed twice with distilled water, and transferred to the 1.2% PVA medium. After a week of incubation, the concentration of the residual PVA was estimated. One of these isolates, designed strain P7, slightly degraded PVA (Fig. 2A). To identify the bacterial species, almost 16S rRNA gene was amplified by PCR using previously described primers, 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGTGTGTACAAGGC-3')⁴. Sequencing was performed using BigDye Terminator ver. 3.1 and ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). 16S rRNA sequence of strain P7 was compared with those from the other type strains using the Ribosomal Database Project (<http://rdp.cme.msu.edu/index.jsp>). As the results, the 16S rRNA gene sequence of strain P7 showed high similarity to that of *Novosphingobium capsulatum* GIFU11526 (Fig. 3A). Cells of strain P7 were yellow-pigmented and rod-shaped bacteria (data not shown). These features supported that strain P7 was a member of genus

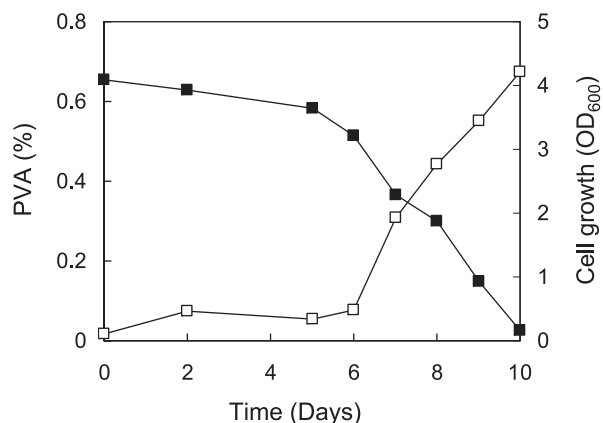


Fig. 1. PVA concentration and bacterial growth in the PVA medium. The microorganisms from activated sludge were inoculated into 0.6% PVA medium and incubated with shaking at 30°C. Residual PVA concentration (closed squares) and cell growth (open squares) were measured by the method described in context.

Novosphingobium (Sphingomonas). The nucleotide sequence of the 16S rRNA gene from the strain P7 has been deposited in DDBJ/EMBL/GenBank databases under accession no. AB512109.

Although strain P7 showed obvious PVA-degrading activity, growth and PVA degradation of strain P7 in PVA medium were limited compared with that of activated sludge (Fig. 2A). It was considerable that strain P7 received some growth factors or coenzymes for degradation of PVA from the other bacteria. To identify the co-factor for PVA degradation by strain P7, the modified PVA medium (M-PVA medium), which did not contain yeast extract but trace metals, was used. First, strain P7 was inoculated into the M-PVA medium containing 0.1% glucose as a sole carbon source. However, cell growth was not observed after a week of incubation (data not shown). This feature showed that strain P7 requires the growth factor, which was not present in the M-PVA medium. Therefore, we added various nutrients to the M-PVA medium. When the 0.8% M-PVA medium was supplied with 0.1% peptone, PVA was degraded completely by strain P7 after a week of incubation (Fig. 2B). Thus, each of twenty kinds of amino acids, which were main components of peptone, was added to the M-PVA medium at the final concentration of 0.01%. One of twenty amino acids, only addition of methionine induced the growth and PVA degradation of strain P7 (Fig. 2B). Strain P7 could also grow in the M-PVA medium containing 0.1% glucose and 0.01% methionine (data not shown). These results showed that methionine worked as the growth factor for strain P7. Large number of PVA-degrading bacteria required exogenous pyrroloquinoline quinone (PQQ) as a coenzyme for PVA-degrading enzyme. However, strain P7 did not require supply of PQQ externally. It was assumed that strain P7 produced PQQ by itself or had PQQ-independent enzymes for PVA degradation.

Strain P7 did not grow and degrade PVA in the M-PVA medium, but did in the activated sludge. It was assumed

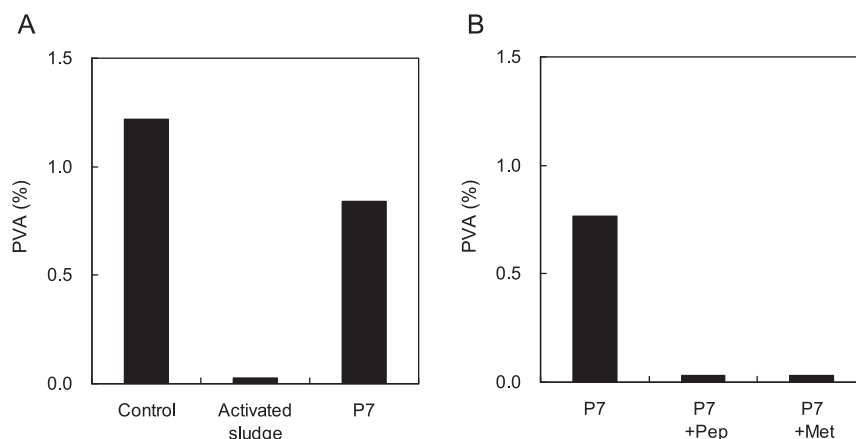


Fig. 2. Degradation of PVA in the PVA minimal medium by strain P7 and activated sludge.

(A) Strain P7 or activated sludge was inoculated into the 1.2% PVA medium. (B) Strain P7 was inoculated into 0.8% M-PVA medium with or without 0.1% peptone (Pep) and 0.01% methionine (Met). Bacterial cultures were incubated with shaking at 30°C and residual PVA concentration was measured after a week of incubation by the method described in context.

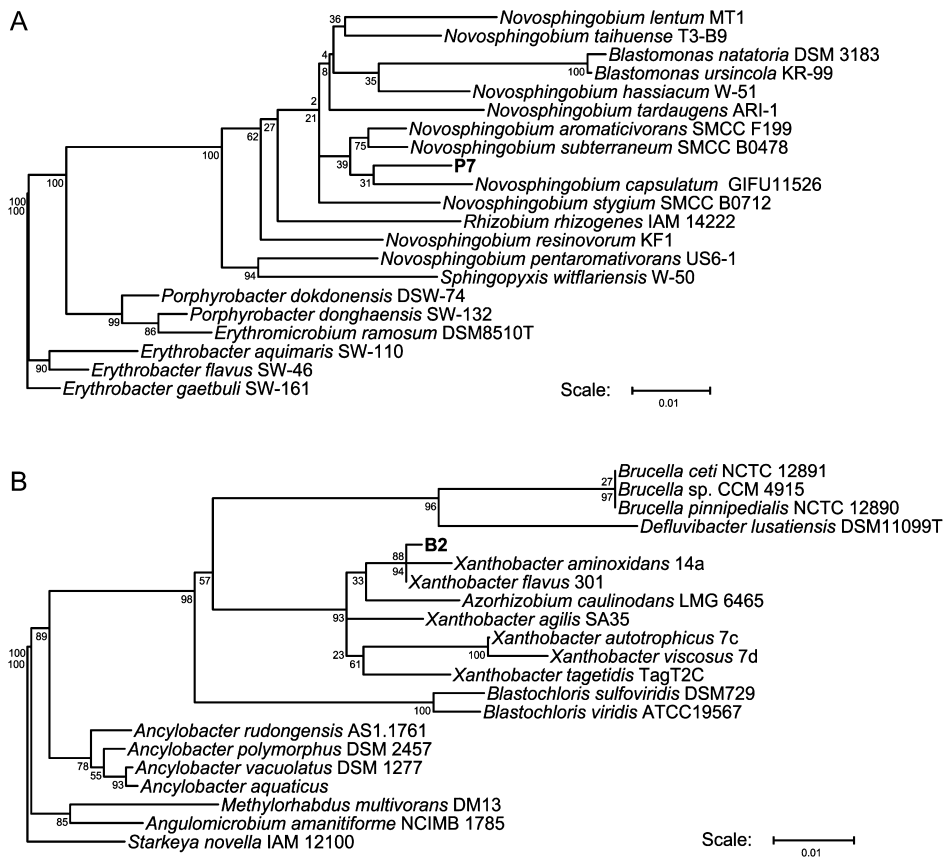


Fig. 3. Neighbor-joining trees of the 16S rRNA gene sequences.

PVA-degrading strain P7 (A) and its symbiotic strain B2 (B) were isolated from activated sludge. Bacterial isolates in this study were shown by bold style. The scale bar represented 0.01 substitutions per nucleotide position. Bootstrap values (from 100 replicates) were indicated at the nodes.

that the putative symbiotic bacteria might support growth of strain P7 and degradation of PVA by strain P7. To identify the symbiotic bacteria from the activated sludge, PVA-degrading activated sludge were diluted and spread on LB agar plates. After forming colonies, eight isolates and strain P7 were grown in the LB medium. Bacterial cells were collected from 1 ml cultures by centrifugation, and washed and resuspended with distilled water. The cell suspensions of strain P7 and each isolates were co-inoculated into 1.2% M-PVA medium. After a week of incubation, only co-culture of strain P7 with one isolate named strain B2 could degrade PVA was almost completely (Fig. 4). Interestingly, strain B2 could not degrade PVA by itself with or without the amino acids (data not shown). To identify the bacterial strain of B2, 16S rRNA sequence of strain B2 was compared with those from the other type strains. As the results, the 16S rRNA gene sequence of strain B2 showed high similarity to that of *Xanthobacter flavus* 301 (Fig. 3B). The nucleotide sequence of the 16S rRNA gene from the strain B2 has been deposited in DDBJ/EMBL/GenBank databases under accession no. AB512110. Strain P7 requires the supply of methionine, but did not either PQQ or the other exogenous co-factors. Thus, it was assumed that PVA was degraded by these two strains, in which strain B2 produced a growth factor and strain P7 utilized this

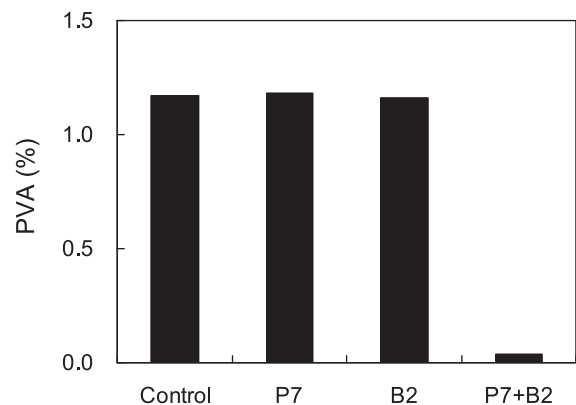


Fig. 4. Degradation of PVA in pure and mixed cultures of strain P7 and B2.

Bacterial strains were inoculated into 1.2% M-PVA medium and incubated with shaking at 30°C. After a week of incubation, residual PVA concentration was measured by the method described in context.

growth factor for PVA degradation. Previously reported PVA-degrading *Sphingomonas* sp., which related to strain P7, was also required PQQ and supplied PQQ with surrounding symbiotic bacteria¹⁾. Our study demonstrated the new type symbiotic degradation of PVA by strain P7 and B2.

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