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Production of Polyvinyl Alcohol Oxidase by a Symbiotic Mixed Culture

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Production of polyvinyl alcohol (PVA) oxidase by *Pseudomonas* sp. strain VM15C, a PVA degrader of a symbiotic PVA-utilizing mixed culture, was examined in various cultures. Despite the absence of PVA in the culture in nutrient broth, VM15C showed approximately the same productivity of PVA oxidase activity as that in the culture with PVA as the sole carbon source, whereas the productivity in the culture with glucose was lower than that of either the nutrient broth or the PVA culture. PVA oxidase activity produced in the nutrient broth culture was predominantly present in the cells, and most of the activity appeared to be in the cytoplasm. In contrast, in the culture with PVA as the sole carbon source, the activity was present in the culture fluid in a larger ratio than in the nutrient broth culture. Thus, production of PVA oxidase activity by this strain was constitutive and repressible, although localization of the produced activity was changed by growth conditions.

In a previous paper (11), Sakazawa et al. reported the finding of symbiotic utilization of polyvinyl alcohol (PVA) by bacterial mixed cultures. They obtained various PVA-utilizing mixed cultures from soil and other environmental sources and isolated the mixed cultures component strains, which were difficult to isolate by usual plate culture techniques, by application of the most-probable-number method. Each PVA-utilizing community could be reconstructed essentially from two kinds of component bacteria which could not utilize PVA in respective axenic cultures. *Pseudomonas putida* VM15A and *Pseudomonas* sp. VM15C are such a pair. VM15C is a strain producing PVA-degrading enzyme, and VM15A supplies a growth stimulant for VM15C in the mixed culture on PVA.

In studies on the roles of these strains in PVA utilization, Sakazawa et al. (11) found that VM15C produces PVA-degrading enzyme despite the absence of PVA. This suggests that VM15C produces PVA-degrading enzyme constitutively. In contrast, Suzuki et al. (12) estimated that PVA-degrading enzyme was inducibly produced by *Pseudomonas* O-3, which was assumed to utilize PVA in axenic culture. This disagreement led us to examine the production of PVA-degrading enzyme by VM15C, a PVA degrader of a symbiotic mixed culture. In this paper, we describe the constitutive production and localization change of PVA oxidase shown in various cultures of the strain.

MATERIALS AND METHODS

Media and growth conditions. The basal medium and the nutrient broth used throughout this work were the same as described previously (11). PVA medium or glucose medium was made by adding 5 g of PVA (Wako Pure Chemical Industry, Osaka, Japan; degree of polymerization, 500; degree of saponification, 88.7%) or 5 g of glucose, respectively, as the sole carbon source, to 1 liter of the basal medium. Cultivation was carried out in 200 ml of medium in a 500-ml shaking flask under continuous reciprocal shaking at 30°C. Growth was monitored by measuring optical density at 660 nm, and dry weight of cells was estimated from the calibration curve.

Microorganisms. *P. putida* VM15A and *Pseudomonas* sp. VM15C were maintained in pure cultures on nutrient agar slants and were used throughout this work.

Preparation of cell extract. Approximately 40 mg (dry weight) of cells was harvested from culture fluid by centrifugation at 15,000 × *g* for 20 min at 4°C. The cells were washed twice with 50 mM phosphate buffer (pH 7.5) and then resuspended in 30 ml of the buffer. The washed cells were disrupted with a sonic oscillator (Kaijo-denki Co., Tokyo, Japan) at 19 kHz for 10 min at 1 to 10°C, the cell debris was removed by centrifugation at 15,000 × *g* for 30 min at 4°C, and the resultant supernatant was used as the cell extract.

Ultracentrifugation of cell extract. The sonic extract of VM15C cells grown on nutrient broth for 5 days was further centrifuged at 100,000 × *g* for 90 min with a Hitachi 55P-2 centrifuge at 4°C. The resultant supernatant was used as the cytosol fraction, and the resultant pellet was suspended in 50 mM phosphate buffer (pH 7.5) and used as the particulate fraction.

Enzyme assay. The assay mixture for PVA oxidase activity contained 150 μmol of phosphate buffer (pH 7.5), 0.25 μmol of 4-aminoantipyrine, 3.2 μmol of phenol, 18 U of peroxidase (EC 1.11.1.7; Sigma Chemical Co., St. Louis, Mo. Type I), 22.5 mg of PVA (Nippon Synthetic Chemical Industry, Osaka, Japan;

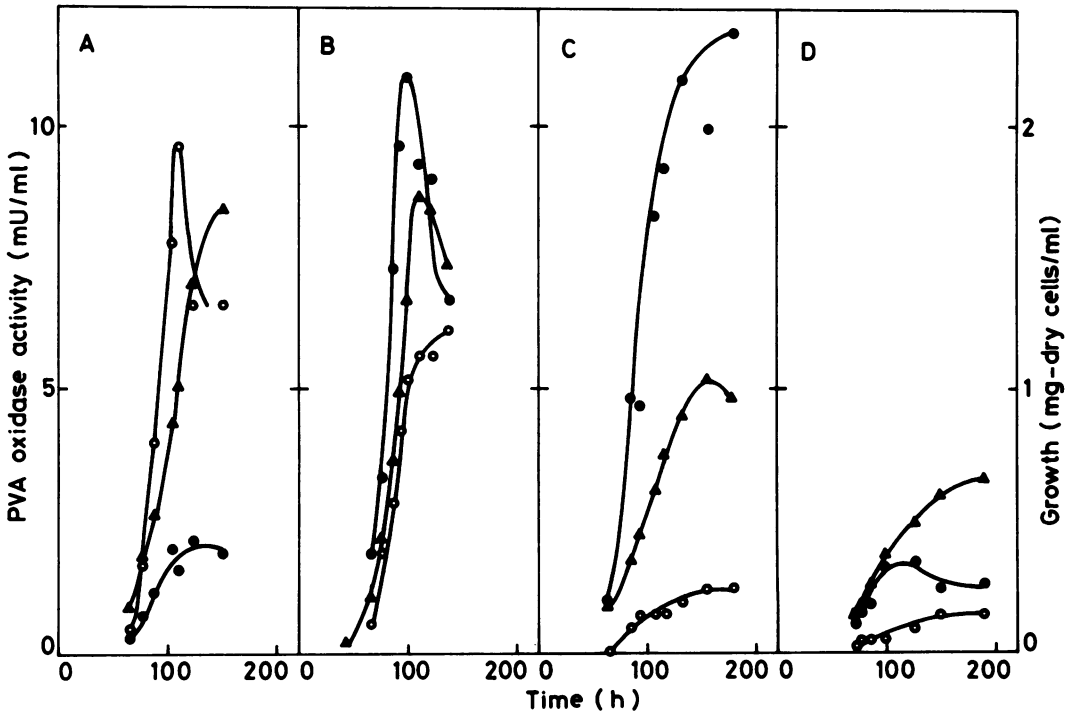

FIG. 1. Time courses of production of PVA oxidase activity in various cultures of VM15C. Cultivation was carried out in 200 ml of each medium in a 500-ml shaking flask. The PVA oxidase activity of cells was estimated by assaying the sonic extract of cells. (A) Mixed culture of VM15C with VM15A in PVA medium; (B) VM15C in PVA medium supplemented with 200 mg of crude factor A per liter; (C) VM15C in nutrient broth; (D) VM15C in glucose medium. Symbols: ○, activity in culture supernatant; ●, activity in cells; ▲, growth.

degree of polymerization, 1,350; degree of saponification, 86.5%), and enzyme preparation in a total volume of 3 ml. The generation of H_2O_2 was followed at 30°C by the increase in absorbance at 500 nm resulting from oxidative coupling of H_2O_2, 4-aminoantipyrine, and phenol (1) with a Shimadzu UV200 spectrophotometer. One unit of PVA oxidase activity was defined as the amount which produced 1.0 μmol of H_2O_2 per min under the assay conditions.

The assay mixture for viscosity-decreasing activity contained 1 mmol of phosphate buffer (pH 7.5), 100 mg of PVA (the same as that used for the PVA oxidase assay), and enzyme preparation in a total volume of 20 ml. The reaction was carried out at 30°C with reciprocal shaking, and the viscosity of the reaction mixture was measured with an Ostwald-type viscometer at 30°C. One unit of viscosity-decreasing activity was defined as the amount which decreased the specific viscosity of the reaction mixture by 10%/h under the assay conditions, according to Sakai et al. (10).

Estimation of protein. The protein content was estimated by the method of Lowry et al. (5).

Preparation of crude factor A. As described previously (11), VM15A produces a growth stimulant (factor A) for VM15C which enables VM15C to grow on PVA. The crude growth stimulant was prepared as follows. VM15A was cultivated in 1 liter of medium containing 10 g of glucose and 2 g of $(NH_4)_2SO_4$ in 1 liter of the basal medium (vitamins omitted) in a 2-liter flask for 5 days with continuous shaking. After cultivation, cells were removed by centrifugation. Five liters of the culture supernatant was passed through a charcoal column (2.5 by 30 cm). The column was washed successively with water and 50% ethanol, followed by elution with 300 ml of 50% ethanol containing 1.4% NH_4OH. The eluate was dried in vacuo at 50°C. About 0.3 g of a crude substance was obtained and used as crude factor A. It was added to PVA medium for cultivation of VM15C in an axenic culture on the medium (see legend to Fig. 1).

Materials. PVA was used after purification as described previously (11). Other chemicals used were of reagent grade.

RESULTS

Producer of PVA oxidase. Previously we decided that VM15C is a PVA degrader for PVA utilization in a mixed culture with VM15A and that VM15A does not produce PVA-degrading enzyme by measuring the viscosity-decreasing activity of each axenic culture grown on nutrient broth (11). Recently, Watanabe and co-workers isolated and purified PVA oxidase, which was characterized as a secondary alcohol oxidase (7), and oxidized PVA-degrading enzyme (10) from a PVA-utilizing bacterial culture and showed that PVA was degraded by successive

TABLE 1. Production of PVA oxidase by component strains of a symbiotic PVA-utilizing mixed culture

Strain	Growth (mg of dry cells/ml)[a]	Activity (mU)			
		Viscosity-decreasing		PVA oxidase	
		Supernatant[b]	Cell[c]	Supernatant[b]	Cell[c]
VM15A	1.09	ND[d]	ND	ND	ND
VM15C	0.89	24	133	0.9	12.3

[a] Cultivation was carried out in 200 ml of nutrient broth in a 500-ml shaking flask. The cultivation periods were 54 h for VM15A and 132 h for VM15C.
[b] Activity per milliliter of culture supernatant.
[c] Activity per milligram of dry cells. The activity of cells was estimated by assaying the sonic extract of cells.
[d] ND, Not detected.

reactions of these enzymes. In a mixed culture of VM15A and VM15C, if PVA degradation is carried out in the same way, the viscosity-decreasing activity is detectable only for a strain producing both types of the enzyme. For a strain producing only PVA oxidase, the viscosity-decreasing activity would not be detected. To define the roles of these symbionts precisely and to examine whether PVA oxidase participates in the PVA degradation by the mixed culture, the PVA oxidase activity of the axenic culture of each strain grown in nutrient broth was examined together with viscosity-decreasing activity (Table 1). For the culture of VM15C, both activities were detected for cells and culture supernatant, but there was no activity in the culture of VM15A. This showed that VM15C produces PVA oxidase, but VM15A does not. PVA oxidase is thought to participate in PVA degradation by VM15C.

Production of PVA oxidase activity. The time courses of production of PVA oxidase activity were examined during growth of VM15C in three kinds of medium: PVA medium, nutrient broth, and glucose medium (Fig. 1). Cultivation of VM15C in PVA medium was carried out in two ways: in a mixed culture with VM15A and in an axenic culture in medium supplemented with crude factor A. PVA oxidase activity was assayed for cell extract and supernatant prepared from each culture as described in Materials and Methods. VM15C produced various amounts of PVA oxidase activity in either cells or supernatants of these cultures. In the mixed culture, the PVA oxidase activity produced was present predominantly in the culture fluid. In contrast, the activity was present mainly in the cells in the culture in PVA medium supplemented with crude factor A, nutrient broth, or glucose medium.

Relationships between growth and total PVA oxidase activity produced (the sum of the activities of cells and culture supernatant) at early stages of these cultivations were compared (Fig. 2). Linear relationships were obtained for all of these cultures, except for the mixed culture. For this culture, the relationship was nonlinear; however, above approximately 0.4 mg of dry cells per ml of growth, linearity was obtained. The slope of each line shows the productivity of PVA oxidase activity (ratio of production rate of PVA oxidase activity and cell mass) in the respective culture of VM15C. The slope of the linear part of the line for the mixed culture may show the productivity of VM15C in the mixed

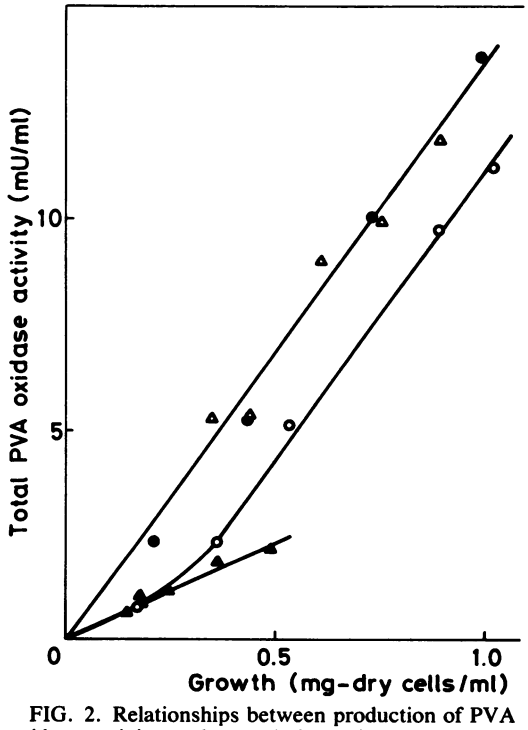

FIG. 2. Relationships between production of PVA oxidase activity and growth in various cultures of VM15C. The data of Fig. 1 were replotted in this figure. Total PVA oxidase activity is the sum of the activity of the cells and that of the culture supernatant. Symbols: ○, mixed culture of VM15C with VM15A in PVA medium; ●, VM15C in PVA medium supplemented with 200 mg of crude factor A per liter; △, VM15C in nutrient broth; ▲, VM15C in glucose medium.

TABLE 2. Distribution of PVA oxidase activity in fractions obtained by ultracentrifugation of cell extract[a]

Enzyme prepn	Total activity (mU)[b]	Sp act (mU/mg of protein)
Cell extract	304	24.1
Cytosol fraction	237	22.1
Particulate fraction	61	30.3

[a] Cell extract of VM15C was centrifuged at 100,000 × *g* for 90 min.
[b] Total activity contained in each preparation.

culture, because that strain grows to such an extent that the cell mass of VM15A in the mixed population is negligible (11). Alternatively, the low productivity at the initial growth stage of the mixed culture may have resulted from the presence of PVA oxidase-nonproducing strain VM15A. The productivity of VM15C in the nutrient broth culture was approximately equal to that in either the axenic culture or the mixed culture in PVA medium (approximately 14 mU/mg of dry cells), and that in the glucose medium culture was lowest (approximately 5 mU/mg of dry cells) (Fig. 2).

Intracellular localization of PVA oxidase activity. With regard to the cells of VM15C grown on nutrient broth for 5 days, intracellular localization of PVA oxidase activity was examined. The cells were osmotically shocked by the method of Anraku and Heppel (2). Approximately 5% of the initial activity of cells was released into the fluid by this treatment, and most of the activity was retained in the shocked cells. Also, most of the activity was not released from cells by the lysozyme (EC 3.2.1.17) treatment of Osborn et al. (8). This suggested that most of the PVA oxidase of the cells is not located in the periplasmic space.

The sonic extract of the cells was fractionated by centrifugation at 100,000 × *g* for 90 min, and the PVA oxidase activities of the resultant particulate fraction and cytosol fraction were assayed (Table 2). Approximately 80% of the initial activity was present in the cytosol fraction, and a little activity was detected in the particulate fraction. This suggested that most of the PVA oxidase activity of the cells is present in the cytoplasm, with part of the activity located in the membranous matrix.

Apparent inducible production of PVA oxidase. No or poor activity of PVA oxidase was produced in a mixed culture of VM15A and VM15C in nutrient broth. As described above, PVA oxidase activity was produced abundantly in the mixed culture in PVA medium. Thus, in the mixed culture PVA oxidase was produced as if it were an inducible enzyme. This phenomenon seems to result from a difference between the mixed populations in nutrient broth and in PVA medium. As described previously (11), VM15A grows much faster than does VM15C in axenic cultures in nutrient broth. Also, VM15A seems to predominate in mixed culture in nutrient broth, differing from the mixed culture in PVA medium. Therefore, no or poor production of PVA oxidase seems to occur in the mixed culture in nutrient broth.

DISCUSSION

In this work we have shown some characteristics of production of PVA oxidase by a PVA degrader of a symbiotic mixed culture. We found that VM15C produces PVA oxidase activity abundantly in nutrient broth, despite the absence of PVA, and that the produced activity is predominantly present in cells. Such abundant production of PVA-degrading enzyme in the absence of PVA and cellular accumulation of the enzyme by microorganisms are phenomena that have not been reported before. Suzuki et al. (12) presumed that a PVA-utilizing strain, *Pseudomonas* O-3, produces PVA-degrading enzyme inducibly from the observation that the cells grown in carbon sources other than PVA could not degrade and oxidize PVA. In the culture in nutrient broth, VM15C showed approximately the same productivity of PVA oxidase activity as that in the cultures with PVA as the sole carbon source, and in the culture in glucose medium, it showed lower productivity than those. This shows that production of PVA oxidase activity by VM15C is repressible, but constitutive. The low productivity during growth with glucose seems to result from catabolite repression.

It was suggested that most of the PVA oxidase activity of the cells grown in nutrient broth is located in the cytoplasm, and not in the periplasmic space. The periplasmic space is external to the cytoplasmic membrane, and periplasmic enzymes are exoenzymes which are secreted through the cytoplasmic membrane as well as extracellular enzymes in the culture fluid. It is thought that in nutrient broth, VM15C retains most of the produced PVA oxidase intracellularly without secretion or release of the enzyme.

With regard to the PVA oxidase activity released into the culture fluid, if productivities of the activity are estimated as in Fig. 2, those of VM15C in the mixed culture with VM15A in PVA medium, in axenic cultures in PVA medium supplemented with crude factor A, and in axenic cultures in nutrient broth are approximately 12, 4, and 1 mU/mg of dry cells, respectively. Thus, VM15C showed larger productivities of released activity in the cultures with PVA than in the culture in nutrient broth. This may result from secretion or release of PVA oxidase

which was accumulated in the cells in the case of growth in nutrient broth, because VM15C showed approximately equal productivity of total PVA oxidase activity. If this is so, induction of secretion or release of PVA oxidase into the culture fluid, but not induction of synthesis of the enzyme, may occur in the cultures with PVA. Studies on secretion or release of the enzyme are now in progress. PVA oxidase secreted or released into the culture fluid and the enzyme retained in the cells may be linked through such a precursor-product relationship as those estimated between membrane-bound and extracellular penicillinase (EC 3.5.2.6) of *Bacillus licheniformis* (9, 13) or between internal or membrane-bound and external invertase (β-D-fructofuranosidase, EC 3.2.1.26) of *Saccharomyces* cells (3, 6).

For PVA utilization, it would be necessary to degrade the molecules outside the cells, in the culture fluid, or at the cell surface because it is a macromolecule, and uptake of it would be impossible without degradation. The PVA oxidase activity produced by VM15C in the axenic culture in PVA medium supplemented with crude factor A was present mainly in cells, although a considerable amount of the activity was present in the culture fluid. This shows that such a predominant presence of PVA oxidase in the culture fluid as that observed in the mixed culture with VM15A is not essential for PVA utilization by VM15C and may suggest that PVA oxidase of cells participates in PVA degradation at the cell surface. Kawai et al. (4) reported that in a polyethylene glycol (a water-soluble polymer)-utilizing bacterial culture, essentially all of the degrading enzyme (polyethylene glycol dehydrogenase) activity was present in the cells, and not in the culture supernatant.

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