Enhancement of Pyrroloquinoline Quinone Production and Polyvinyl Alcohol Degradation in Mixed Continuous Cultures of *Pseudomonas putida* VM15A and *Pseudomonas* sp. Strain VM15C with Mixed Carbon Sources

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In a mixed continuous culture of *Pseudomonas putida* VM15A and *Pseudomonas* sp. strain VM15C with polyvinyl alcohol (PVA) as the sole source of carbon, growth of the PVA-degrading bacterium VM15C and, hence, PVA degradation were limited by the growth factor, pyrroloquinoline quinone, produced by VM15A. Feeding of a carbon source for VM15A, ethanol, with PVA enhanced pyrroloquinoline quinone production and caused increases in the VM15C population and PVA degradation in a mixed continuous culture. There was an optimum range for PVA degradation of the ethanol concentration, although pyrroloquinoline quinone concentrations in continuous mixed cultures increased with increasing ethanol concentration.

In preceding studies (5, 7-11), polyvinyl alcohol (PVA) was shown to be utilized by mixed cultures of pairs of bacterial symbionts which did not grow on PVA in axenic cultures. *Pseudomonas putida* VM15A and *Pseudomonas* sp. strain VM15C are such a pair. VM15C metabolizes PVA, whereas VM15A produces the growth factor (trivial name, factor A) essential for this PVA utilization. In continuous mixed cultures of the symbionts with PVA as the sole source of carbon, it was found that factor A becomes the growth-limiting substrate for the PVA degrading, VM15C, and hence the rate-limiting factor for PVA degradation with increasing dilution rate (7). On the basis of this, PVA degradation should be increased on enhancement of the production of factor A, as well as on addition of factor A. VM15A produces factor A with many kinds of substances as the carbon source. Thus, there was the possibility that provision of an additional carbon source for VM15A with PVA might increase PVA degradation by the mixed cultures. Recently, we identified factor A as pyrroloquinoline quinone (PQQ) and showed that axenic cultures of VM15C degrade PVA when provided with PQQ (11). PQQ is a novel coenzyme which was discovered among methanol dehydrogenases of methylotrrophic bacteria (2, 6). This paper reports enhancement of PVA degradation and PQQ production in mixed continuous cultures with mixed carbon sources.

**MATERIALS AND METHODS**

**Materials.** Authentic PQQ, which was purified from the culture supernatant of a methylotrrophic bacterium, was a kind gift from M. Ameyama, Yamaguchi University, Yamaguchi, Japan (1). PVA (average molecular weight, 2,000; Aldrich Chemical Co., Inc., Milwaukee, Wis.) was saponified in 0.8 N NaOH at 30°C at a concentration of 8% (degree of saponification, 99.9%). The PVA was precipitated in 90% acetonitrile and washed with methanol in a funnel and then in a Soxhlet extractor.

**Microorganisms and growth conditions.** An Acinetobacter calcoaceticus mutant lacking d-glucose dehydrogenase activity was the same as that previously described (11), which was kindly supplied by M. Ameyama. Glucose-BTB agar was composed of 1% glucose, 1% peptone, 0.04% bromothymol blue, and 1.5% agar (pH 7.3).

*P. putida* VM15A and *Pseudomonas* sp. strain VM15C, maintained in pure cultures on nutrient agar slants, were used throughout this work. The basal medium was the same as that described previously (5). Unless otherwise noted, the saponified PVA was used as a carbon source in the basal medium at a concentration of 2.5 g/liter. Batch cultivation was carried out with 200 ml of medium in 500-ml flasks at 30°C with reciprocal shaking. Chemostat cultivation was carried out with a Bioflo C-30 (New Brunswick Scientific Co., Edison, N.J.) at a dilution rate of 0.064/h at 30°C as previously described (7). The biomass was estimated turbidometrically from the optical density at 660 nm. The PVA concentration and cell numbers of the two strains were measured as previously described (7). The ethanol concentration was determined with a gas chromatograph (GC8A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a glass column (0.26 by 200 cm) packed with Gaskuropack 54 (Gasukuro Kogyo Inc., Tokyo, Japan). The temperatures of the column and inlet were maintained at 120 and 140°C, respectively. The flow rate of nitrogen carrier gas was 60 ml/min. The culture supernatant (10 ml) was directly injected into the column. The PQQ concentration in the culture supernatants was determined by a biological assay.

**Biological assay.** Culture supernatant (10 to 30 ml) of a steady-state mixed continuous culture was adjusted to pH 2.0 with dilute HCl and then passed through a Sep-Pak C18 cartridge (Waters Associates, Inc., Milford, Mass.). After the cartridge was washed with 20 ml of dilute HCl (pH 2.0), PQQ was eluted with 10 ml of methanol-water (7:3, vol/vol). The eluate was dried under reduced pressure and then dissolved in 0.1 to 0.5 ml of water. The PQQ content was determined by a paper disk method with the *A. calcoaceticus* mutant, which is unable to form PQQ but produces an apoglucone dehydrogenase requiring PQQ as the coenzyme in its membrane. Each plate was prepared by laying 250 ml of glucose-BTB agar on a glass plate (21 by 26 cm) on a 200-ml, 2% agar bed in a stainless steel bath (24 by 30 cm). The mutant was spread and cultivated at 30°C for 24 h on the plates. Paper disks (6 mm) immersed in sample and authentic

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PQQ solutions were placed on each plate culture, followed by further incubation for 24 h at 30°C. The diameters of the yellow circles, resulting from the pH decrease due to the formation of gluconic acid around the disks, were calibrated from the linear relationship between them and the log PQQ concentration (300 to 2,500 ng/ml) in the authentic solutions.

RESULTS

Effect of mixed carbon sources on mixed batch cultures. Mixed batch cultivation of P. putida VM15A and Pseudomonas sp. strain VM15C were carried out with mixed carbon sources of PVA and several substances which are utilized by VM15A (Table 1). Of the additional carbon sources used with PVA, glucose and peptone were utilized, but ethanol, acetate, and glycerol were not utilized by VM15C. Biomass production and PVA degradation were enhanced by the use of these mixed carbon sources. Since the enhancement of PVA degradation and that of biomass production with ethanol were greatest, ethanol was selected as the additional carbon source in further experiments. In axenic batch cultures with PQQ, it was confirmed that ethanol did not stimulate growth of VM15C on PVA.

Effect of ethanol on mixed continuous cultures. Ethanol was added to the feed medium at a concentration of 0.1 g/liter for a steady-state mixed continuous culture of P. putida VM15A and Pseudomonas sp. strain VM15C established with PVA as the sole source of carbon (Fig. 1). The cell density and PVA degradation rapidly increased, and a new steady state was established after a transient state of about 100 h. Residual PVA at the new steady state was approximately 0.01 g/liter. Increased VM15A and VM15C populations and PQQ concentration were observed in the new steady state. The PQQ concentration increased from 1.3 to 20 ng/ml between the two steady states. Figure 2 shows the results for steady-state mixed cultures established with ethanol (at concentrations of 0 to 1.0 g/liter) and PVA. The VM15A population and PQQ concentration increased with the ethanol concentration. Ethanol was almost completely consumed in the mixed cultures (<0.005 g/liter). PVA degradation and VM15C populations in the continuous mixed cultures with mixed carbon sources were greater than in the culture with

<table>
<thead>
<tr>
<th>Carbon source used with PVA</th>
<th>Growth (OD660)</th>
<th>Residual PVA (g/liter)</th>
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<tbody>
<tr>
<td>Sodium acetate</td>
<td>0.554</td>
<td>0.337</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.975</td>
<td>0.188</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.604</td>
<td>0.588</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.715</td>
<td>0.250</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.910</td>
<td>0.257</td>
</tr>
<tr>
<td>None</td>
<td>0.194</td>
<td>1.12</td>
</tr>
</tbody>
</table>

* Carbon sources were used at a concentration of 0.1 g/liter. The cultivation time was 98 h.
* OD660, Optical density at 660 nm.

FIG. 1. Effect of ethanol on a mixed continuous culture of P. putida VM15A and Pseudomonas sp. strain VM15C. At the time indicated by the arrow, ethanol was added to the feed medium to 0.1 g/liter. Symbols: ○, optical density at 660 nm; ●, PVA; □, PQQ; △, VM15A population; ▲, VM15C population.

FIG. 2. Effect of ethanol concentration in feed medium on steady-state mixed continuous cultures of P. putida VM15A and Pseudomonas sp. strain VM15C. Symbols: ○, optical density at 660 nm; ●, PVA; □, PQQ; △, VM15A population; ▲, VM15C population.
PVA as the sole source of carbon but decreased somewhat as the ethanol concentration increased. The optimum range of the ethanol concentration for PVA degradation was found to be 0.1 to 0.25 g/liter.

DISCUSSION

In a mixed culture of P. putida VM15A and Pseudomonas sp. strain VM15C, the microbial interaction, i.e., provision of the growth factor, PQP, supports PVA utilization by VM15C. Feeding of ethanol with PVA enhanced the production of PQP and caused increases in the VM15C population and PVA degradation in a mixed continuous culture. Results of axenic batch culture experiments showed that ethanol itself did not stimulate PVA utilization by VM15C. PVA degradation by VM15C was enhanced indirectly by a carbon source for VM15A, ethanol, via production of PQP by VM15A. PVA degradation can be enhanced without PQP in the case of mixed cultures. Thus, a regulatory means, involving a microbial interaction, was found for enhancing PVA degradation in mixed continuous cultures.

The saturation constant (K_s) of PQP for VM15C was estimated to be approximately 0.7 ng/ml from the axenic growth rate on PVA (11). The PQP concentration in the mixed continuous culture with ethanol at 0.1 g/liter was 20 ng/ml, about 30-fold the K_s. Growth limitation of VM15C by PQP was thought to be overcome in mixed continuous cultures with ethanol at more than 0.1 g/liter.

PVA degradation and VM15C populations, however, decreased as the ethanol concentration increased in the feed medium, despite the increase in PQP concentration in mixed continuous cultures. Although the cause of this phenomenon is not known at present, the increase in ethanol concentration may lead to enhancement of harmful interactions such as competition for a certain nutrient and production of a growth inhibitor for VM15C, as a result of the increase in the VM15A population, together with the enhancement of PQP production. It is generally accepted that some interactions occur simultaneously in a mixed culture (3).

If bacterial symbionts similar to VM15A and VM15C are used or participating in PVA wastewater treatment with activated sludge systems (4), the following possibilities are suggested from the results of this work: (i) some carbon sources other than PVA in wastewater may act as stimulants for PVA degradation, (ii) PVA degradation may be enhanced by addition of a carbon source, and (iii) the amounts of such carbon sources may have to be controlled to obtain stable PVA degradation.

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LITERATURE CITED