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Production of (R)-(-)-mandelic acid by electrochemically driven enzyme bioreactor

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ABSTRACT

Enterococcus faecalis was cultivated under oxidative conditions established by adding some oxidants. FAD and lipoic acid either stimulate the biosynthesis of benzoylformate reductase or stabilize the enzyme, while MV^{2+} enhance the biosynthesis of the oxidoreductase but destabilize it. Since MV^{2+} destabilize the benzoylformate reductase, substituting FAD for MV^{2+} as a redox mediator would be desirable. Production of (R)-(-)-mandelic acid by a coupled reaction between the enzymatic reaction using benzoylformate reductase and the electrocatalytic reduction under the conditions of 1.5 U LiDH ml^{-1}, 0.2 mM FAD, and 0.3 mM NAD^+ is now performing.

INTRODUCTION

Enzymatic reductions of carbonyl groups have received attention in the applications for the synthesis of chiral compounds. Hydorxy acids, amino acids, and alcohols has been utilized in the food industry and served as chiral building blocks for the synthesis of pharmaceuticals, flavors, and agrochemicals. (R)-(-)-Mandelic acid is used as a precursor for semisynthetic penicillin and cephalosporin plus many other pharmaceuticals. Although there have been a number of studies to prepare R-(-)-mandelic acid, the practical production of R-(-)-Mandelic acid was often hampered by low opical purity and/or yield, complex processes et cetera.

In this study, a new electroenzymatic method for the production of (R)-(-)-mandelic acid was tried. Enzymatic reaction of oxidoreductase can be coupled with electrode reduction of NAD^+ in which electron transfer reagent and enzyme are used to electron transfer between electrode and NAD^+. Since methyl viologen(MV^{2+}) is very toxic and represent an undesirable contaminant in substance prepared using MV^{2+}, an alternative solution that would totally avoid the use of the toxic redox mediator would be attractive. In advance, the effect of oxidative stress on cell growth and biosynthesis of enzyme (tentatively named bezoylformate reductase) were examined.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The *Enterococcus faecalis* culture was streaked on TSB agar plates and grown at 37 °C overnight. Starter cultures were prepared by inoculating 8 ml of TSB medium with a single colony and incubated at 37 °C for 4.5 hr on a rotary shaker (180 rpm). Main cultivation was performed by inoculating 100 ml of TSB medium in a 250 ml flask with 3 ml of the starter culture and incubating at 37 °C on a rotary shaker (180 rpm). Cells were counted using Bioscreen C (Labsystem, Oy, Helsinki, Finland) or harvested by centrifugation at 4 °C. The harvested pellets were washed with 0.1 M phosphate buffer, pH 7.0 and suspended (1:2 w/v) in 15 M phosphate buffer (pH 6.3) containing 2 mM 2-mercaptoethanol and distrupted by glass beads (150~212 microns). The suspensions were clarified by centrifugation and the enzyme in the supernatants was assayed as described below.

The enzymatic activity

The enzymatic activity was evaluated from the absorbance decrease at 340 nm followed by the oxidation of NADH in a spectrophotometer (Shimadzu UV-1601 : Shimadzu Co., Japan). Assay solutions were prepared by mixing 2.5 ml of 0.1 M phosphate buffer (pH7.5), 0.2 ml of 83 mM sodium benzoylformate, and 100 μl of the crude enzyme solution. The mixture kept at 30 °C for 5 min, and then the enzyme reaction was started by adding 50 μl of 13 mM NADH.

Procedure for the production of (R)-(-)-mandelic acid

Electrochemical reduction of NAD^+ was performed as was in our previous report[1]. A plate (6 cm^2) of gold amalgam was used as a working electrode and a platinum plate as a counter electrode. Ag/AgCl reference electrode was immersed into the cathode compartment filled with 0.5 M phosphate (pH 7.0) electrolyte containing NAD^+ (0.3 mM), FAD (0.2 mM), lipoamide dehydrogenase (1.5 U/ml), bezoylformate reductase (3.0 U/ml), and sodium bezoylformate (50.0 mM). After the electrolyte was purged with N_2 gas for 30 min, the electrolysis was started using a potentiostat (HA-501, Hokuto Denko Co., Japan). Electrolysis was carried out at the constant potential of -0.7 V vs. Ag/AgCl electrode at 25 °C.

Assays

(R)-(-)-Mandelic acid and benzoylformate were analyzed with HPLC (Shimadzu CBM 10A, Japan), using an μ Bondapack C 18 column (3.9×300 mm) with an UV detector (235 nm). A mobile phase of 10 % CH_3OH was used.

RESULTS AND DISCUSSION

Variation of cell growth and enzyme biosynthesis characteristics under oxidative stress

Bacterial cells are able to sense and adjust to a great number of different environmental stresses including reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. These species produced by the incomplete reduction of oxygen during respiration or by exposure to light, metals, radiation, and oxidation-reduction active compounds(oxidants) such as MV^{2+} and menadion. Exposure to these cause the induction of predominantly unique sets of proteins. FAD and lipoic acid, biomolecules, in addition to MV^{2+} were selected as the oxidants[2].

The oxidants affected rarely the cell growth at the concentrations below 10 μM, implying that the oxidants might be nontoxic to the cell. In contrast to the cell growth, enzyme biosynthesis was significantly stimulated under the oxidative cultivations as shown in Fig. 1. By the supplementation of MV^{2+}, the enzymatic activity was increased with the higher concentrations of each oxidant and reached the maximum value at the concentration of 4 μM , over which the activity decreased. However, the enzymatic activity increased under the oxidative stress by FAD or lipoic acid and each maximum value reached at the concentration of 2 μM did not changed by adding more mount of each oxidant. Fig. 2 exhibits the enzymatic activity under the oxidative cultivation conditions established by supplementing 4 μM of oxidants at which each oxidant exerted a stronger influence. Under the oxidative stress by FAD or lipoic acid, the higher enzymatic activity was measured regardless of cultivation time. In the case of MV^{2+}, the enzymatic activity was increased during the 6 h cultivation, but decreased with the longer cultivation. Therefore, it could be postulated that FAD and lipoic acid either stimulate the biosynthesis of the benzoylfomate reductase or stabilize the enzyme, while MV^{2+} enhance the biosynthesis of the oxidoreductase but destabilize it.

Construction of the electrochemically drivern enzyme bioreactor

In our previous study[3], the mediated electrocatalytic reduction of NAD^+ was proposed to be optimal under the conditions of 1.5 U diaphorase ml^{-1}, 0.2 mM MV^{2+}, and 0.3 mM NAD^+. The supposed optimal conditions of the three species were conformed by performing a coupled reaction between the electrocatalytic reduction of NAD^+ and an enzymatic reduction in the presence of benzoylformate reductase. The conversion yield of (R)-(-)-mandelic acid from 50 mM benzoylformate under the conditions of the three species described previously, was about 50%. Such a low conversion yield was considered to be caused by

the inactivation of benzoylfomate reductase by the toxic $MV^{2+}$and so an alternative solution that would totally avoid the use of the toxic redox mediator should be taken into account. Application of the natural redox cofactor would be advisable. FAD is not only nontoxic to benzoylformate reductase but also an essential cofactor for LiDH. Therefore substituting FAD and LiDH for MV^{2+} and diaphorase, respectively would be desirable. We are now performing the production of (R)-(-)-mandelic acid by a coupled reaction between the enzymatic reaction of benzoylformate reductase and the electrocatalytic reduction under the conditions of 1.5 U LiDH ml^{-1}, 0.2 mM FAD, and 0.3 mM NAD^+.

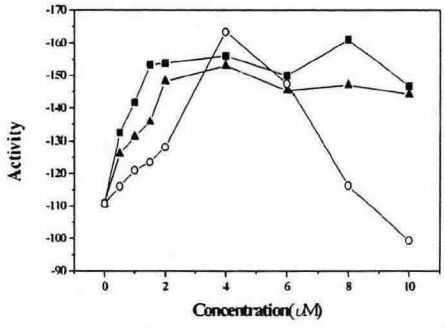

Fig.1 Effect of oxidants on the enzymatic activity. The enzymatic activities were assayed after the 6h of cultivation. Each oxidants was added after 1h of cultivation. Symbols: ○, methyl viologen;▲, lipoic acid; ■, FAD.

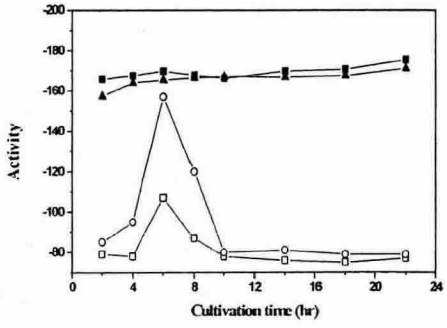

Fig.2 Effect of oxidants the enzymatic activity. Each oxidant (4μM) was added after 1h of cultivation. Symbols: □, control; ○, methyl viologen; ▲, lipoic acid; ■, FAD.

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