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Note

Synthesis of (*R*)-1,3-Butanediol by Enantioselective Oxidation Using Whole Recombinant *Escherichia coli* Cells Expressing (*S*)-Specific Secondary Alcohol Dehydrogenase

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The synthesis of (*R*)-1,3-butanediol (BDO) from its racemate was studied using whole cells of recombinant *Escherichia coli* expressing an (*S*)-specific secondary alcohol dehydrogenase (CpSADH) from *Candida parapsilosis* by enantioselective oxidation. Under the optimized conditions, the yield of (*R*)-1,3-BDO reached 72.6 g/l, with a molar recovery yield of 48.4% from a racemate of 15% and an optical purity of 95% *ee*.

Key words: secondary alcohol dehydrogenase; (*R*)-1,3-butanediol; NAD^+-regeneration; enantioselective oxidation

(*R*)-1,3-butanediol (BDO) is an optically active alcohol used as an intermediate for the production of pharmaceuticals such as azetidinone derivatives.[1] We are studying a method for the practical synthesis of (*R*)-1,3-BDO from the more inexpensive racemate by enantioselective oxidation, rather than from the corresponding ketone, 4-hydroxy-2-butanone (4H2B) by asymmetric reduction. In our previous reports, we showed that *Candida parapsilosis* IFO 1396 cells produced (*R*)-1,3-BDO by the enantioselective oxidation of (*S*)-1,3-BDO[2] and that the enantioselective oxidation was catalyzed by an (*S*)-specific secondary alcohol dehydrogenase (CpSADH).[3] Furthermore, we reported the cloning and expression of the *CpSADH* gene and the preliminary synthesis of (*R*)-1,3-BDO with recombinant *E. coli* cells expressing CpSADH.[4] In this paper, we describe the practical production of (*R*)-1,3-BDO under optimal conditions.

E. coli cells expressing CpSADH for the synthesis of (*R*)-1,3-BDO were prepared by the following method. *E. coli* W3110 (IFO 12713) cells, harboring a high expression plasmid for CpSADH, pSE-CPA1,[5] were grown in 100 ml of a 2×YT medium (Bacto-Tryptone, 20 g/l; Bacto-Yeast extract, 10 g/l; NaCl, 10 g/l; pH 7.2) containing ampicillin (50 mg/l) in a 500-ml baffled shake flask at 30°C on a rotary shaker (140 rpm) to the optical density at 600 nm (OD_{600}) of 3–4; after the addition of 2% lactose as an inducer, the culture medium was further shaken for 11 h. The cells harvested by centrifugation were used as a catalyst.

The synthesis of (*R*)-1,3-BDO was done at 30°C in the standard reaction mixture (25 ml) containing 100 mM potassium phosphate buffer (pH 6.8), racemic 1,3-BDO, and cultured cells obtained from 25 ml of the medium with shaking at 145 strokes per min in a 500-ml Sakaguchi flask. The amounts of 1,3-BDO and 4H2B were measured by gas chromatography under the following conditions: column, PoraPak PS (Waters Corporation, Milford, MA, U.S.A.); column temperature, 165°C; carrier gas, N_2; detection, flame ionization detector. The optical purity of 1,3-BDO was measured as 1,3-BDO diacetyl by chiral HPLC with a Chiralcel OB packed column (4.6× 250 mm, Daicel Chemical Industries, Ltd., Tokyo, Japan) at 40°C, eluted with *n*-hexane:2-propanol (19:1) at a flow rate of 1 ml/min, and detected at 220 nm.

The pH of a reaction mixture containing cultured cells obtained from 3.8 ml of the medium was optimized in the range of 6.5–8.0, as lysis and foaming in reaction mixtures at pH above 8.0 were observed and prohibited exact analyses. As shown in Fig. 1, after the incubation of 17 h, there was little difference in the optical purities of residual 1,3-BDO among pH 6.5–8.0; after an incubation of 40 h, alternatively, the reaction at pH 6.8 gave the highest optical purity (88.6% *ee* (*R*)). These findings suggested that the rate-limiting step of enantioselective oxidation was a regeneration step of the coenzyme, NAD^+, and the optimal pH and/or the stablest pH for the regeneration of the coenzyme was around pH 6.8, since the optimal pH of CpSADH for the oxidation of (*S*)-1,3-BDO was 9.0 and CpSADH was stable within the range of 8.0–11.0.[3]

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Abbreviations: 4H2B, 4-hydroxy-2-butanone; BDO, butanediol; CpSADH, (*S*)-specific secondary alcohol dehydrogenase of *Candida parapsilosis*; *ee*, enantiomer excess

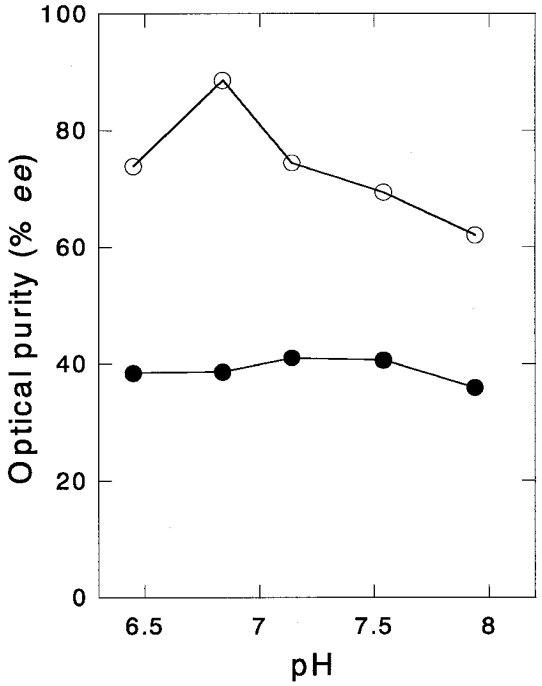

Fig. 1. Effects of pH on the Synthesis of (*R*)-1,3-BDO.

The reaction mixture (25 ml), containing 100 mM potassium phosphate buffer (pH 6.5–8.0), 5% 1,3-BDO, and cultured cells obtained from 3.8 ml of the medium, was shaken in a 500-ml Sakaguchi flask at 140 strokes per min and 30°C for 40 h. The optical purity of (*R*)-1,3-BDO after an incubation of 17 (●) and 40 (○) h was measured by chiral HPLC.

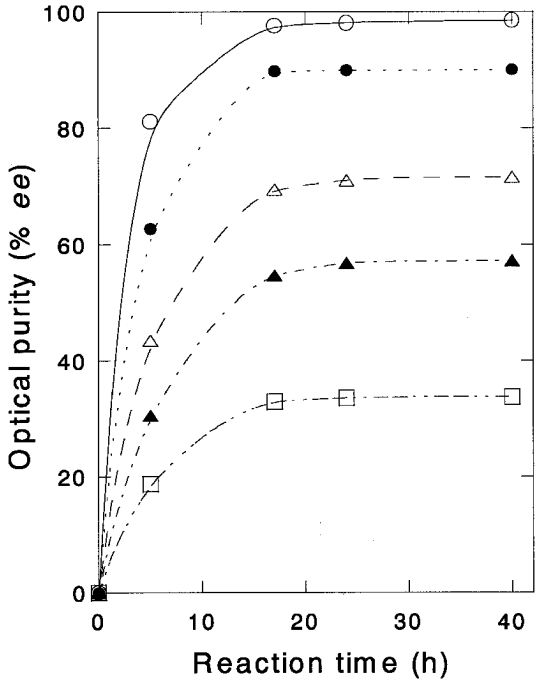

Fig. 2. Effects of the Concentrations of 1,3-BDO on Enantioselective Oxidation.

The reaction mixture (25 ml), containing 7–15% 1,3-BDO and cultured cells obtained from 25 ml of the medium, was incubated at pH 6.8. Other reaction conditions and analytical methods were the same as those given in Fig. 1. 1,3-BDO concentrations: 7% (○), 10% (●), 12% (△), 15% (▲), 20% (□).

Secondly, the effects of the concentrations of 1,3-BDO in the reaction mixture were studied. At the reaction containing 7% 1,3-BDO, the optical purity of (*R*)-1,3-BDO reached 97.6% *ee* after incubation of 17 h, as shown in Fig. 2. However, in the reactions containing higher concentrations of 1,3-BDO, the optical purities of (*R*)-1,3-BDO did not increase from a 17-h to a 40-h incubation. To elucidate the reason for the cessation of enantioselective oxidation in the reaction mixtures containing 10% 1,3-BDO, the residual CpSADH activity of the cell-free extract and the (*R*)-1,3-BDO-producing activity of *E. coli* cells were measured. After a 17-h incubation, *E. coli* cells retained a CpSADH activity of 64.5% but had no 1,3-BDO-producing activity, as shown in Table 1. These findings suggested that the cessation of enantioselective oxidation resulted from the loss of NAD^+ and/or the NAD^+-regeneration activity of the cell. To overcome the loss of NAD^+ and/or NAD^+-regeneration activity, several adducts were tested, such as acetone, sodium hydrogen sulfite, and several components of the medium. Acetone (20 g/l-reaction mixture) was expected to function as a co-substrate to regenerate NAD^+ by CpSADH itself, and sodium hydrogen sulfite (57.8 g/l-reaction mixture) was expected to reduce the inactivation of NAD^+-regeneration activity by 4H2B. The addition of acetone or sodium hydrogen sulfite, against our expectations, caused a decrease of the optical purity. Alternatively, YT medium (Bacto-Tryptone, 10 g; Bacto-Yeast extract, 5 g; NaCl, 5 g in a reaction mixture) was ex-

Table 1. Effects of the Incubation in the Reaction Mixture Containing 10% 1,3-BDO on the Residual Activities of CpSADH and the 1,3-BDO-producing Activity

Treatment	CpSADH (%)	Specific 1,3-BDO-producing activity (% *ee*/OD_{600}/h)
None	100	1.90
After reaction	64.5	0
After storage	81.4	1.76

The reaction mixture (25 ml), containing 10% 1,3-BDO and cultured cells obtained from 25 ml of the medium, was shaken at 30°C for 17 h. Other reaction conditions were the same as those given in Fig. 1. The cells were collected by centrifugation and washed with saline, and the CpSADH activity of the cell-free extracts and the (*R*)-1,3-BDO-producing activity of the cells were measured. The CpSADH activity was measured as follows: the collected cells were suspended in 50 mM Tris-HCl (pH 9.0) and 0.02% 2-mercaptoethanol, and disrupted with a Bioruptor UCD-200 (Cosmo BIO Co., Ltd., Tokyo, Japan). The supernatant was obtained by centrifugation at 16,000 *g* for 10 min. The CpSADH was assayed spectrophotometrically.[3] One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per min at 30°C. (*R*)-1,3-BDO-producing activity was measured as follows: A reaction mixture (25 ml) containing 5% 1,3-BDO and collected cells (45 OD_{600}) was incubated in a 500-ml Sakaguchi flask at 30°C for 17 h with reciprocal shaking (245 strokes/min). The specific (*R*)-1,3-BDO-producing activity was expressed as the percent change of optical purity, per OD_{600} at the start of the reaction and per hour of the reaction time (% *ee*/OD_{600}/h). None, no treatment; after storage, after storage at 4°C for 17 h instead of the synthetic reaction of (*R*)-1,3-BDO.

pected to increase and maintain an amount of NAD^+ in cells and/or NAD^+-regeneration activity of cells by growth of cells. The addition of YT medium to a reaction mixture, as expected, brought about an increase in the turbidity of the reaction mixture at 600 nm after a 17-h incubation and an increase of the optical purity of (*R*)-1,3-BDO from 89.8% to 96.1% after a 41-h incubation. The addition of Bacto-Yeast extract alone could replace that of YT-medium with effect, but the identification of the effective components in Bacto-Yeast extract and the reasons for the effects upon the addition of YT-medium remains to be discovered.

Furthermore, in the reaction containing 15% 1,3-BDO, cells obtained from the 25-ml culture medium needed to be added to the reaction mixture after the 17-h incubation to reach the optical purity of 95% *ee*.

The asymmetric reduction of carbonyl compounds is quite a significant method for the production of optically active alcohol.[6,7] Preparative application of the asymmetric reduction method requires an efficient regeneration system of the coenzyme, NAD(P)H, since *E. coli* cells do not have a sufficient amount.[8,9] Kataoka *et al.*[10] and Kizaki *et al.*[11] published reports about an efficient production system using *E. coli* cells co-expressing a carbonyl reductase and glucose dehydrogenase from *Bacillus megaterium* as a catalyst in an *n*-butyl acetate/water biphasic system. Alternatively, enantioselective oxidation is also a useful and practical method to produce optically active compounds, such as alcohols,[12] diol,[13] and hydroxy acids.[14] There are, nevertheless, only a few reports on efficient production systems of optically active compounds using recombinant *E. coli* cells expressing an enantioselective oxidase or dehydrogenase. In this report, we have established a practical method to produce (*R*)-1,3-BDO from an inexpensive racemic 1,3-BDO with a high optical purity by enantioselective oxidation using whole recombinant cells expressing CpSADH without the addition of an expensive coenzyme, NAD^+.

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