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Cloning and Expression in *Escherichia coli* of a Gene Coding for a Secondary Alcohol Dehydrogenase from *Candida parapsilosis*

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A gene encoding a stereo-specific secondary alcohol dehydrogenase (CpSADH) that catalyzed the oxidation of (*S*)-1,3-BDO to 4-hydroxy-2-butanone was cloned from *Candida parapsilosis*. This CpSADH-gene consisted of 1,009 nucleotides coding for a protein with M_r 35,964. A recombinant *Escherichia coli* JM109 strain harboring the expression plasmid, pKK-CPA1, produced (*R*)-1,3-BDO (93.5% ee, 94.7% yield) from the racemate without any additive to regenerate NAD^+ from NADH.

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

(*R*)-1,3-BDO is an important chiral compound as an intermediate for the production of pharmaceuticals such as azetidinone derivatives.[1] We have been investigating industrial production systems for (*R*)-1,3-BDO and have reported previously the establishment of an efficient microbial production of (*R*)-1,3-BDO from the racemate by *Candida parapsilosis*.[2]

Furthermore we purified and characterized (*S*)-1,3-BDO dehydrogenase, which catalyzed the stereo-specific oxidation of (*S*)-1,3-BDO to 4-hydroxy-2-butanone.[3] The enzyme had a higher activity for secondary alcohols than for primary alcohols, and showed high stereo-specificity for (*S*)-forms of several secondary alcohols (such as 2-butanol, 2-octanol, 1-phenylethanol and methyl 3-hydroxybutyrate). From these properties, (*S*)-1,3-BDO dehydrogenase was found to be a novel stereo-specific secondary alcohol dehydrogenase (SADH).

To study the molecular structure and function of CpSADH and to construct a CpSADH-producing system for the practical synthesis of (*R*)-1,3-BDO, we have attempted to clone and characterize the gene encoding CpSADH from *Candida parapsilosis*. In this paper, we described the cloning, sequencing, and expression of the gene encoding CpSADH (designated *CpSADH*) and the synthesis of (*R*)-1,3-BDO using a recombinant *Escherichia coli* expressing *CpSADH*.

Materials and Methods

Bacterial strains, plasmids, and cultivation conditions. *Candida parapsilosis* was used as an CpSADH-gene donor strain. The host strain of *E. coli* used was JM109 (*hsdR17*, *recA1*, *del*(*lac-proAB*), *endA1*, *gyrA96*, *thi-1*, *relA1*, *supE44*, [F′, *traD36*, *proAB*, *lacI*^{q}, *lacZdelM15*]). The vector plasmids used in this study were pUC18 (Takara Shuzo, Kyoto, Japan) and pKK223-3 (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

E. coli was grown in LB medium (1% Bacto-Tryptone (Difco), 0.5% Bacto-Yeast extract (Difco), and 1% NaCl (pH 7.2)). This media was solidified with 15 g of agar, if necessary. Selective antibiotic medium contained ampicillin (50 μg/ml).

Amino-terminal and inner amino acid sequence analysis. CpSADH was purified from the culture supernatant of *C. parapsilosis* by the method described in our previous paper.[3] The purified enzyme (3.8 nmol) was digested for 6 h with lysyl endopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan), in the molar ratio of 200 (CpSADH):1 (lysyl endopeptidase) at 30°C in 0.1 M Tris-HCl buffer (pH 8.5) containing 4 M urea. The digested peptide fragments were put on a reverse-phase HPLC column (TSK gel ODS-120T, 4.6×250 mm, Tosoh Co., Ltd., Tokyo, Japan), and eluted with a linear gradient of 0–60% acetonitrile over a period of 60 min at flow rate of 1.0 ml/min. The amino acid sequences of these peptide fragments and N-terminal region of the purified CpSADH were analyzed with a gas-phase protein sequencer (Model 477A-120A, Perkin-Elmer Applied Biosystems Division, Foster City, USA).

Recombinant DNA techniques. The standard molecular biology techniques were done as described by Maniatis *et al.*[4] Chromosomal DNA was prepared from *C. parapsilosis* as described by Cryer.[5]

PCR for the core region of CpSADH gene. Amplification by PCR was done in a reaction mixture (100 μl) containing 50 ng of chromosomal DNA as a template, both

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Abbreviations: 1,3-BDO, 1,3-butanediol; SADH, secondary alcohol dehydrogenase; CpSADH, secondary alcohol dehydrogenase from *Candida parapsilosis*; ScADH1, alcohol dehydrogenase 1 from *Saccharomyces cerevisiae*; ScADH2, alcohol dehydrogenase 2 from *Saccharomyces cerevisiae*; ScADH3, alcohol dehydrogenase 3 from *Saccharomyces cerevisiae*; ScADH5, alcohol dehydrogenase 5 from *Saccharomyces cerevisiae*; ZmADH1, alcohol dehydrogenase 1 from *Zymomonas mobilis*; TbADH, alcohol dehydrogenase from *Thermoanaerobium brockii*

primers (100 pmol each), four deoxyribonucleotide triphosphates (dNTPs, final concentrations, 0.1 mM each), 2 U of AmpliTaq DNA polymerase (Takara Shuzo, Kyoto, Japan) in the buffer for AmpliTaq, consisted of 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin. The reaction was done for 30 cycles (30 s at 94°C, 30 s at 45°C and 2 min at 70°C) with a GeneAmp PCR System 2400 (Perkin-Elmer Applied Biosystems, Foster City, U.S.A.). The DNA fragment amplified by PCR was purified and ligated to the *Sma* I site of pUC18 with a SureClone ligation kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden) to obtain a plasmid, pCPA2.

Inverse PCR for DNA sequences that flank a core region. Chromosomal DNA from *C. parapsilosis* was digested with *Hae* II or *Mfl* I. Digested DNAs were circularized with T4 DNA ligase. Amplifications by inverse PCR[6] were done in a reaction mixture (100 μL) containing 100 ng of circularized DNA obtained as described above, both primers (100 pmol each), four dNTPs (final concentrations, 0.1 mM each), and 2 U of AmpliTaq DNA polymerase in the buffer for AmpliTaq. The reaction was done for 30 cycles (30 s at 94°C, 30 s at 50°C and 3 min at 70°C). Amplified DNA fragments derived from *Hae* II- and *Mfl* I-digested chromosomal DNA as a template for inverse PCR were ligated into the *Sma* I site of pUC18 with a SureClone ligation kit to obtain plasmids pCPA4 and pCPA5, respectively.

Nucleotide sequencing. The dideoxynucleotide chain termination method was used for nucleotide sequencing of the cloned DNA fragments with a Dye-terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, U.S.A.). The CpSADH-gene data has been deposited in the DDBJ DNA database under the accession number AB010636. Sequence data were analyzed by GENETYX-WIN (Software Development Co., Ltd., Tokyo, Japan).

Construction of an expression plasmid of CpSADH. Two primers, primer 5 and primer 6, were prepared for PCR to obtain the open reading frame (ORF) of *CpSADH*. Primer 5, 5′-TCGCGAATTCAATGTCAATTCCATCAAGC-3′ consisted of a *Eco*R I site and 21 nucleotides of 5′-end of *CpSADH*; primer 6, 5′-AGATCTTACTATGGATTAAAAACAACTCTAC-3′, was consisted of *Bgl* II site, 19 nucleotides of the 3′-end of the ORF and 2 termination codon. Amplification by PCR was done in a reaction mixture (100 μl) containing 50 ng of the chromosomal DNA of *C. parapsilosis* as a template, both primers (50 pmol each), four dNTPs (final concentrations, 0.1 mM each), and 2 U of AmpliTaq DNA polymerase in the buffer for AmpliTaq. The reaction was done for 30 cycles (30 s at 94°C, 30 s at 45°C and 2 min at 70°C). Amplified DNA fragments were ligated into the *Sma* I site of pUC18 with a SureClone ligation kit to obtain a plasmid, pCPA6R. pCPA6R was digested with *Eco*ICR I, ligated with a *Hind* III linker (CAAGCTTG), digested with *Eco*R I and *Hind* III, and the resultant 1020-bp fragment was ligated to pKK223-3, already digested with *Eco*R I and *Hind* III. The resulting plasmid, designated pKK-CPA1 (Fig. 2), was prepared from the transformed *E. coli* JM109.

Cultivation of E. coli carrying the expression plasmid. E. coli JM109 harboring pKK-CPA1 (designated as *E. coli* JM109 (pKK-CPA1)) were grown in 5 ml of LB broth containing ampicillin (50 mg/l) for 32 h at 30°C. 1 ml of culture broth was used to inoculate 100 ml of 2 × YT broth (Bacto-Trypton, 20 g/l; Bacto-yeast extract, 10 g/l; NaCl, 10 g/l; pH 7.2) and ampicillin (50 mg/l) in a 500-ml baffiedshake flask. Cultures were grown at 30°C for 14 h on a rotary shaker (140 rpm). The cells were harvested by centrifugation at 5200 × *g* for 8 min.

Synthesis of (R)-1,3-BDO from the racemate. E. coli (pKK-CPA1) was grown in 100 ml of 2 × YT medium containing 50 mg/l ampicillin at 30°C for 16 h. Centrifuged cells were added to 25 ml of a reaction mixture consisting of 5% 1,3-BDO in 100 mM potassium phosphate buffer (pH 6.5) in a Sakaguchi flask. The reaction mixture was shaken at 30°C for 24 h.

Enzyme assay and protein assay. The harvested cells were suspended in 50 mM Tris-HCl (pH 9.0), 0.02% 2-mercaptoethanol, and 2 mM phenylmethanesulfonyl fluoride, and disrupted with a Mini-Bead Beater (Biospec Products Inc., USA). The supernatant was obtained by centrifugation at 16,000 × *g* for 10 min and used for the enzyme assay. CpSADH was assayed spectrophotometrically at 30°C. The standard assay mixture contained 50 μmol of (*S*)-1,3-BDO, 2.5 mmol of NAD^+, 50 μmol of Tris-HCl buffer (pH 9), and enzyme in a final volume of 1 ml. 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. Specific activity is expressed as units per milligram of protein. Protein was measured using the protein-dye binding method[7] using bovine serum albumin as a standard.

Measurement of optical purity. The optical purity of 1,3-BDO was measured as 1,3-BDO diacetyl by HPLC, using a column with a chiral stationary phase (Chiralcel OB, 4.6 × 250 mm, Daicel Chem. Co., Tokyo), eluting with hexane: 2-propanol (19:1) at a flow rate of 1 ml/min, and detecting at 220 nm at 40°C.

Measurement of 1,3-BDO and 4H2B. 1,3-BDO and 4H2B were measured by GC (Shimadzu GC-14A, Kyoto, Japan) under the following conditions: column, PoraPak PS (Waters Corporation, Massachusetts, U.S.A.); column temperature, 165°C; carrier gas, N_2; detection, flame ionization detector (FID).

Results and Discussion

N-terminal and inner amino acid sequences of CpSADH and preparation of PCR primers

On the basis of the amino acid sequence of the N-terminal region (MXIPSSQYGFVFNKQSGLNLR-

NDLPXH: X, unknown amino acid), primer 1 (5′-TA(T/C) GGN TT(T/C) GTN TT(T/C) AA(T/C) AA(A/G) CA-3′), which corresponds to Y-8 to Q-15), was synthesized. After digestion of the purified enzyme by lysyl endopeptidase, the digested peptide fragments were separated and sequenced. According to one of the internal partial amino acid sequences (LKELPEYIEKLRNNAYEGRVVFNP), primer 2 (5′-C(G/T) NCC (C/T) TC (A/G)TA NGC (A/G) TT(A/G) TT-3′), which corresponds to N-13 to R-19, was synthesized.

Cloning and nucleotide sequence of CpSADH

The core region of *CpSADH* was cloned by PCR using primer 1 and primer 2 and subcloned into pUC18 to obtain a plasmid, pCPA2. The core region was 970-bp in length. To clone the 5′ and 3′-flanking regions of the core region, appropriate restriction enzymes for inverse PCR were determined by Southern blotting and hybridization procedures using the core region as probe. *Hae* II

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                                                  BbeI
                                                  GGCGCCAATGCAACATCAATAG      -301
GCACAGCATAAAAATAATCTGGAGTTTCACCCACCGTCAAATATAAAAGCTAAAAAAAAGAATGCAACTTAAAACT   -226
ATCAGTTGCATTATTCACAAGGGGGTGTTTGATTAAACGGATTTAGTTTCTAACAAAAGAACGGACTATTATTTG   -151
AAATTTCTTGGGGTAGAACGCTACAACATACCTATAAATATCTGTTGTCGCTCTCCTTTTTAAATGTTTAAAACC    -76
ATATCAATTTTGAAATCTTTAAGATCAACAACTTCAACCTCCCATTACAATTTATCAAGATCTTTATATCGAAGT     -1

ATGTCAATTCCATCAAGCCAGTACGGATTCGTATTCAATAAGCAATCAGGACTTAATTTGAGAAATGATTTGCCT     75
MetSerIleProSerSerGlnTyrGlyPheValPheAsnLysGlnSerGlyLeuAsnLeuArgAsnAspLeuPro     25

GTCCACAAGCCCAAAGCGGGTCAATTGTTGTTGAAAGTTGATGCTGTTGGATTGTGTCATTCTGATTTACATGTC    150
ValHisLysProLysAlaGlyGlnLeuLeuLeuLysValAspAlaValGlyLeuCysHisSerAspLeuHisVal     50

ATTTACGAAGGGTTGGATTGTGGTGATAATTATGTCATGGGACATGAAATTGCTGGAACTGTTGCTGCTGTGGGT    225
IleTyrGluGlyLeuAspCysGlyAspAsnTyrValMetGlyHisGluIleAlaGlyThrValAlaAlaValGly     75

GATGATGTCATTAACTACAAGGTTGGTGATCGTGTTGCCTGTGTCGGACCCAATGGATGTGGTGGGTGCAAGTAT    300
AspAspValIleAsnTyrLysValGlyAspArgValAlaCysValGlyProAsnGlyCysGlyGlyCysLysTyr    100

TGTCGTGGTGCCATTGACAATGTATGTAAAAACGCATTTGGTGATTGGTTCGGATTGGGGTACGATGGTGGGTAT    375
CysArgGlyAlaIleAspAsnValCysLysAsnAlaPheGlyAspTrpPheGlyLeuGlyTyrAspGlyGlyTyr    125

CAACAGTACTTGTTGGTTACTAGACCACGTAACTTGTCTCGTATCCCAGATAACGTATCTGCAGACGTGGCTGCG    450
GlnGlnTyrLeuLeuValThrArgProArgAsnLeuSerArgIleProAspAsnValSerAlaAspValAlaAla    150

GCTTCAACTGATGCTGTATTGACACCATATCACGCAATCAAGATGGCTCAAGTGTCACCAACTTCGAATATCTTG    525
AlaSerThrAspAlaValLeuThrProTyrHisAlaIleLysMetAlaGlnValSerProThrSerAsnIleLeu    175

CTTATTGGTGCTGGTGGATTGGGTGGAAATGCAATTCAAGTTGCCAAGGCATTTGGTGCGAAAGTTACTGTTTTG    600
LeuIleGlyAlaGlyGlyLeuGlyGlyAsnAlaIleGlnValAlaLysAlaPheGlyAlaLysValThrValLeu    200

GACAAAAAAAAGGAGGCTCGTGACCAAGCAAAGAAGTTGGGTGCTGATGCAGTTTATGAAACATTGCCAGAATCC    675
AspLysLysLysGluAlaArgAspGlnAlaLysLysLeuGlyAlaAspAlaValTyrGluThrLeuProGluSer    225

ATTTCTCCTGGCTCTTTTTCAGCATGTTTTGATTTTGTTTCAGTGCAAGCTACATTTGATGTATGTCAAAAGTAT    750
IleSerProGlySerPheSerAlaCysPheAspPheValSerValGlnAlaThrPheAspValCysGlnLysTyr    250

GTTGAACCAAAGGGTGTAATTATGCCCGTGGGACTCGGTGCTCCTAATTTATCGTTTAATTTGGGAGATTTGGCA    825
ValGluProLysGlyValIleMetProValGlyLeuGlyAlaProAsnLeuSerPheAsnLeuGlyAspLeuAla    275

TTGAGAGAAATTCGAATCTTGGGTAGTTTTTGGGGAACTACTAATGATTTGGATGATGTTTTGAAATTGGTTAGT    900
LeuArgGluIleArgIleLeuGlySerPheTrpGlyThrThrAsnAspLeuAspAspValLeuLysLeuValSer    300

GAAGGTAAAGTTAAACCCGTTGTGAGAAGTGCCAAATTGAAGGAATTGCCAGAGTATATTGAAAAATTGAGAAAC    975
GluGlyLysValLysProValValArgSerAlaLysLeuLysGluLeuProGluTyrIleGluLysLeuArgAsn    325

AATGCTTATGAAGGTAGAGTTGTTTTTAATCCATAGAATGGGGGAGAGGCTAGGTTAAGAGGGAGTTGCGGACAT   1050
AsnAlaTyrGluGlyArgValValPheAsnPro***

AGGCTTGGTAATTGTGGAGTTACAGGTTGAGAAAAGGTTTTGGGGTTGATTGTTTTGGAGTTTGGGTAAGGAGAT   1125
TATATAGCAATTGGGGGTTTTTCAATATGACAAGTTTTAATACTAGATGATGTAGCACATGTCTATTTTAGCTCT   1200
AGCTGATACCTTTCAACCACCAGTTGAATTAAACCCAAATGAAACTGATTAGCATCATTTACAAAATCAACAAAA   1275
TTGCCATCAAGTTGGATATACTTGTGAAAATGGCCTAGATGATTCAATTGACAATTGATGCAAAGATTATAAAGC   1350
AACGAGTCAATCAAATCATCAGTTTTATCCCCCTTCGGTAAATGTAGATGCTCAAGTAGTTGATGTATTGCCAAT   1425
GGCGCCAGTGTTTCATGTCGATACCCTTTTAATTGATAAAGTTTACCAAACCATGAGAGAATCAAATCACGTCGT   1500
ATAATCTTGTTTGTCTTTTTCAATTTTGTATTTATCAGATCT
                                  BglII
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Fig. 1. Nucleotide Sequence and Deduced Amino Acid Sequence of *CpSADH* from *Candida parapsilosis*.

The amino acid sequences of N-terminal region and lysyl endopeptidase fragments are underlined. The possible TATA boxes are double underlined and the CAAT sequence is shown by the waved underline. Putative ADH-Zn binding region is dotted underlined. Asterisk, termination codon.

or *Mfl* I-digested chromosomal DNA were used as templates of inverse PCR after self-circularization. Two oligonucleotide primers were synthesized, one complementary to 63 through 81 (primer 3, 5′-TTCGAATCTTGGGTAGTTTTTG-3′) and the other identical to 815 through 836 (primer 4, 5′-CAATTGACCCGCTTTGGGC-3′) of the core region. PCR products obtained by using *Hae* II or *Mfl* I-digested and circularized chromosomal DNA as templates were subcloned into pUC18 and the resultant plasmids were designated as pCPA4 and pCPA5, respectively. The nucleotides of the insert DNA fragments were sequenced. Both sequences were connected with the known sequence of the core region. Figure 1 shows the nucleotide sequence of *CpSADH* along with its flanking region. In this sequence, the initiation codon ATG (position +1) and the termination codon TAG (position 1009) were found. This open reading frame of 1008 nucleotides encoded a polypeptide of 336 amino acid residues with a calculated molecular weight of 35,964. Amino acid sequences obtained from the purified enzyme were found in the open reading frame (underlined in Fig. 1). In several *Candida* species such as *Candida parapsilosis*, CUG, a universal leucine codon, is read as serine;[8] however, the CUG codon is not present in the ORF of *CpSADH*. The conserved residues of the zinc-containing ADH is observed in the deduced amino acid sequence (position 64 to 78).

In addition to the coding region, we have sequenced the promoter region of *CpSADH*. Two possible promoter sequences, TATAA (position −118 to −114 and −259 to −255), and a putative CAAT motif (position −306 to −303) were found upstream of the initiation codon. The 5′-upstrem region cloned, however, was not enough to express *CpSADH* in *Saccharomyces cerevisiae* (data not shown).

Comparison of amino acid sequence with those of other alcohol dehydrogenases[9]

Comparison of the CpSADH sequence with public data bases was done using the FASTA computer algorithm.[10] Alcohol dehydrogenase I from *Zymomonas mobilis* (ZmADH1) showed the highest similarity score to CpSADH. The overall amino acid sequence identity with alcohol dehydrogenases from *Saccharomyces cerevisiae*, ScADH1, ScADH2, ScADH3 and ScADH5, and ZmADH1 were estimated to be 31.3%, 30.5%, 28.0%, 29.9%, and 31.3% identity, respectively. As for substrate specificity, obvious difference between CpSADH and ScADH1 was present; relative activity for isopropanol to ethanol were 6250% and 6%, respectively.[11] The NADP-dependent secondary alcohol dehydrogenase from *Thermoanaerobium brockii* (TbADH) showed less similarity (23.9% identity) to CpSADH than primary alcohol dehydrogenases, such as ScADH1.

Construction of CpSADH expression plasmid and its expression in E. coli

To construct the expression vector of *CpSADH* in *E. coli*, only the ORF was amplified by PCR with primer 5 and primer 6 and ligated with pUC18 opened by *Sma* I to obtain pCPA6R. All plasmids of *E. coli* JM109 transformants checked had the ORF in the opposite direction from the *lac* promoter. The expression vector, pKK-CPA1 was constructed by inserting *CpSADH* between the *tac* promoter and *rrn*B terminator of pKK223-3. The *E. coli* JM109 strain harboring pKK-CPA1, the *E. coli* JM109 (pKK-CPA1), grew very poorly and slowly on LB plates containing ampicillin (50 mg/l), especially in the presence of IPTG. The growth of transformants was improved by lowering the incubation temperature from 37°C to 30°C.

The CpSADH activity was found in *E. coli* JM109 (pKK-CPA1) cultured in the presence of IPTG, but not in the *E. coli* JM109 cells harboring the vector plasmid pKK223-3 cultured under the same conditions. Further *E. coli* JM109 (pKK-CPA1), however, hardly grew after the addition of IPTG. Since the expression of *CpSADH* was thought to be toxic to *E. coli* JM109, culture and expression conditions were investigated. As shown in Table 1, the expression without induction was more suitable to CpSADH. The amount of CpSADH produced without induction by *E. coli* cells carrying the plasmid pKK-CPA1 corresponds to about 0.3% of the total amount of soluble cellular proteins.

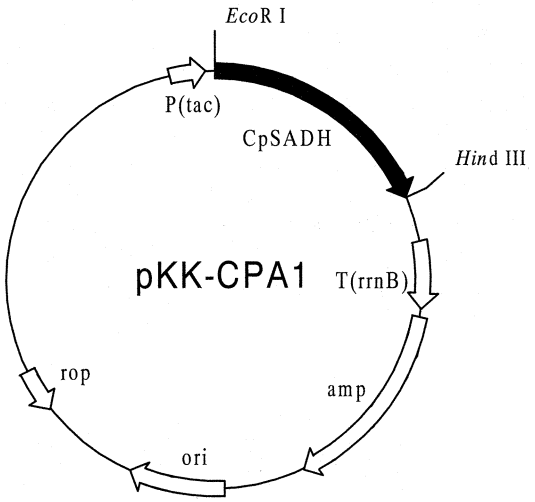

Fig. 2. Restriction Map of pKK-CPA1.
The construction of pKK-CPA1 was described in Materials and Methods.

Table 1. Expression of *CpSADH* in *E. coli* JM109 (pKK-CPA1)

	OD_{600}	CpSADH	
		U/ml-broth	U/mg-protein
With induction	1.34	0.249	0.445
Without induction	6.17	1.28	0.701

In expression with induction, *E. coli* JM109 (pKK-CPA1) was cultured in 2 × YT medium to an optical density (600 nm) of 1.0, and cultured for another 8 h after the addition of 1 mM IPTG. In expression without induction, *E. coli* JM109 (pKK-CPA1) was cultured in 2 × YT medium for 16 h without induction by IPTG.

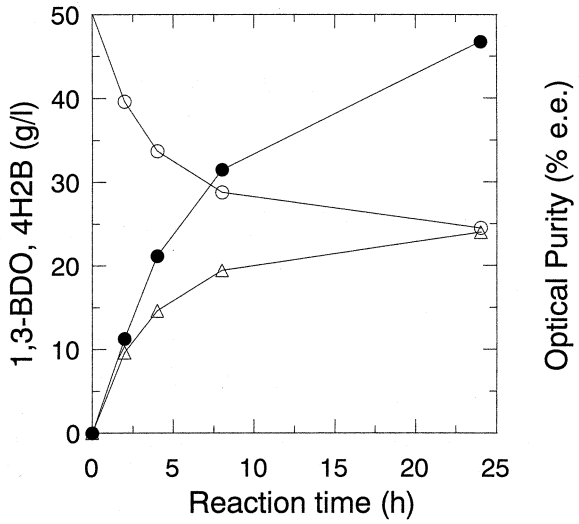

Fig. 3. Time Course of (*R*)-1,3-BDO Production from the Racemate by *E. coli* JM109 (pKK-CPA1).

1,3-BDO (○), 4H2B (△), optical purity (●). The reaction mixture was incubated as described in Materials and Methods.

Synthesis of (R)-1,3-BDO by E. coli expressing CpSADH

E. coli JM109 (pKK-CPA1) was cultivated in LB medium containing ampicillin (50 mg/l) without induction. Cells centrifuged was used to synthesize (*R*)-1,3-BDO from the racemate. The reaction was done in a mixture containing 5% 1,3-BDO at 30°C. As shown in Fig. 3, 5% (555 mM) 1,3-BDO was converted to (*R*)-1,3-BDO (272 mM, 93.5% ee) and 4-hydroxy-2-butanone (272 mM) after 24 h of incubation.

These results showed that asymmetric oxidation using this recombinant *E. coli* strain didn't require an additional NAD^+-regeneration system. NAD^+ as a coenzyme of CpSADH was suggested to be regenerated from NADH by the respiratory pathway in this recombinant *E. coli* strain.

Chiral alcohols have so far mainly synthesized by the asymmetric reduction method, however, this requires an additional NADH- or NADPH-regeneration system such as a glycolytic pathway and glucose dehydrogenase with the corresponding substrate. Recombinant *E. coli* cells expressing the aldehyde reductase gene from *Sporobolomyces salmonicolor* was reported to require exogenous $NADP^+$ and glucose dehydrogenase for the synthesis of ethyl (*R*)-4-chloro-3-hydroxybutyrate from ethyl 4-chloroacetoacetate.[12]

The evidence presented in this report demonstrated the asymmetric oxidation system using recombinant *E. coli* expressing CpSADH is an efficient and convenient system to synthesize chiral alcohols. Further work is needed to prepare cells have higher oxidative activity that contains both CpSADH activity and NADH-regeneration activity, and to accumulate higher concentration of (*R*)-1,3-BDO.

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