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Industrial production of (R)-1,3-butanediol by new biocatalysts

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Abstract

We have developed the economical and convenient biocatalytic process for the preparation of (*R*)-1,3-butanediol (BDO) by stereo-specific microbial oxido-reduction on an industrial scale. (*R*)-1,3-BDO is an important chiral synthon for the synthesis of various optically active compounds such as azetidinone derivatives lead to penem and carbapenem antibiotics.

We studied on two approaches to obtain (*R*)-1,3-BDO. The first approach was based on enzyme-catalyzed asymmetric reduction of 4-hydroxy-2-butanone; the second approach was based on enantio-selective oxidation of the undesired (*S*)-1,3-BDO in the racemate. As a result of screening for yeasts, fungi and bacteria, the enzymatic resolution of racemic 1,3-BDO by the *Candida parapsilosis* IFO 1396, which showed differential rates of oxidation for two enantiomers, was found to be the most practical process to produce (*R*)-1,3-BDO with high enantiomeric excess and yield.

We characterized the (*S*)-1,3-BDO dehydrogenase purified from a cell-free extract of *C. parapsilosis*. This enzyme was found to be a novel secondary alcohol dehydrogenase (CpSADH). We have attempted to clone and characterize the gene encoding CpSADH and express it in *Escherichia coli*. The CpSADH activity of a recombinant *E. coli* strain was more than two times higher than that of *C. parapsilosis*. The production yield of (*R*)-1,3-BDO from the racemate increased by using the recombinant *E. coli* strain. Interestingly, we found that the recombinant *E. coli* strain catalyzed the reduction of ethyl 4-chloro-3-oxo-butanoate to ethyl (*R*)-4-chloro-3-hyroxy-butanoate with high enantiomeric excess. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: (*R*)-1,3-Butanediol; *Candida parapsilosis*; (*S*)-1,3-Butanediol dehydrogenase; Secondary alcohol dehydrogenase

1. Introduction

Optically active 1,3-butanediol (1,3-BDO) is an important material for synthesizing various optically active compounds such as azetidinone derivatives, which are intermediate material for antibiotics [1,2], pheromones [3], fragrances [4,5], and insecticides [6] (Scheme 1). (*R*)-1,3-BDO is especially a starting material of azetidinone derivatives, important intermediates in the synthesis of penem and carbapenem antibiotics, etc. for industrial uses. (Scheme 2).

2. Screening of microorganisms producing optically active 1,3-BDO from 4-hydroxy-2-butanone (4H2B) by asymmetric reduction

Although microbial asymmetric reduction processes for optically active 1,3-BDO have been reported by Bakers' yeast, the productivity was not

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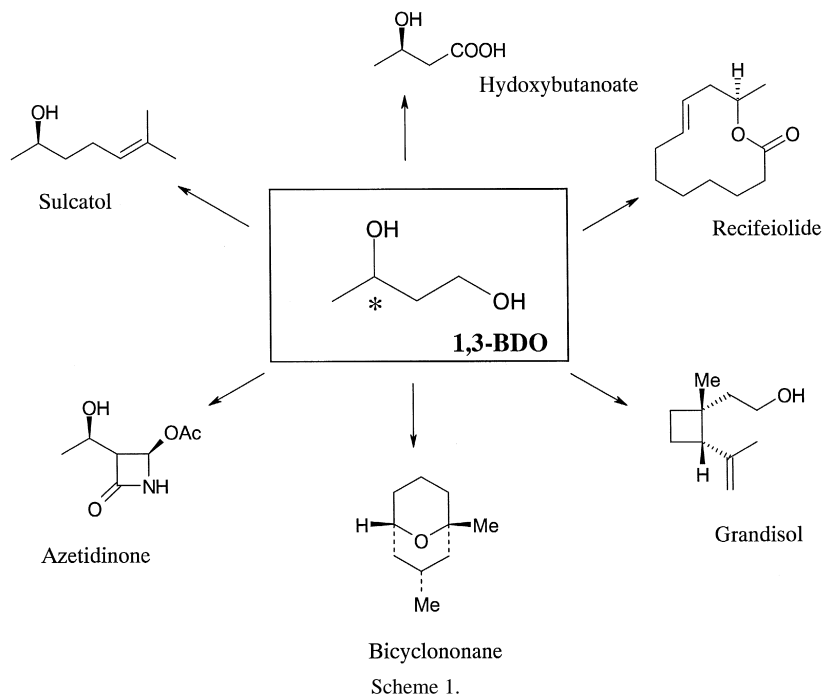
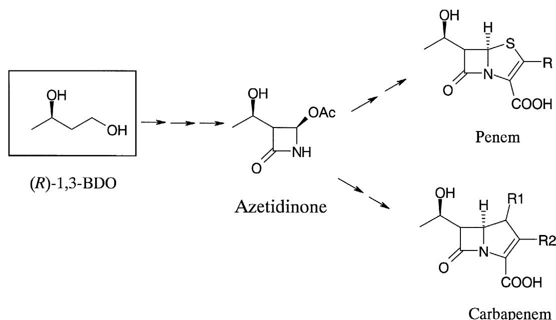


Scheme 1.

satisfactory, and they produced only (*R*)-1,3-BDO [8,9]. Thus, we started a program to establish an economical and convenient microbial process from 4H2B. All microorganisms used were type cultures preserved in our laboratories. The method of screening is shown in Table 1. Many microorganisms have been found to produce optically active 1,3-BDO from 4H2B. Eleven strains produced (*R*)-1,3-BDO, two strains produced (*S*)-1,3-BDO with high optical purity. *Candida arborea* IAM 4147 and *Issatchenkia scutulata* IFO 10070 converted 4H2B to (*R*)-1,3-BDO with 99% e.e., but in low yield. We chose *Kluyveromyces lactis* IFO 1267 as the best strain for producing (*R*)-1,3-BDO, because it produced in high yield. *C. parapsilosis* IFO 1396 was the best strain for (*S*)-1,3-BDO production. *K. lactis* IFO 1267 produced (*R*)-1,3-BDO with 93% e.e. [7] (Table 1).



Scheme 2.

3. Screening of microorganisms producing optically active 1,3-BDO from the racemate

Enantio-selective oxidation of the primary alcohol of 1,3-BDO to optically active 3-hydroxybutyric acid was reported [11], but there was no report on enantio-selective oxidation of 1,3-BDO to 4H2B. Thus, we screened microorganisms. The method of screening is shown in Table 2. As a result of screening, we have found that many yeast, fungi and bacteria strains

Table 1
Screening of microorganism producing optically active 1,3-BDO from 4H2B by asymmetric reduction

Microorganisms (yeasts, bacteria, fungi)
↓
Cultivation (100 ml / 500 ml flask, 30°C, 24-48 h with shaking)
↓
Centrifugation and washing with saline
↓
Cells
↓
100mM KPB (pH 7.0) 5 ml, 12% sucrose
↓
Preincubation, 30°C, 10 min.
← 4H2B 50 mg
↓
Incubation (in test tube, 30°C, 48h with shaking)
↓
Centrifugation
↓
Supernatant (2 ml) → Saturation with NaCl → Extraction with EtOAc (2 ml) → Evaporation *in vacuo* → Acetylation with AcCl → HPLC Analysis, Determination of optical purity, Column: Chiralcel OB (Daicel)
↓
GLC Analysis
Determination of formed 1,3-BDO and residual 4H2B
Column: Thermon 3000
Temp: 120°C

Strain	Absolute configuration	%*e.e.*	Formed 1,3-BDO yield (%)
Candida utilis IFO 1086	*R*	81	88
Candida utilis IAM 4246	*R*	85	82
Candida utilis IAM 4277	*R*	95	82
Candida arborea IAM 4147	*R*	99	37
Kluyveromyces lactis IFO 1903	*R*	92	88
Kluyveromyces lactis IFO 1267	*R*	93	99
Hansenula fabianii IFO 1254	*R*	67	16
Hansenula polymorpha ATCC 26012	*R*	85	87
Issatchenkia scutulata IFO 10070	*R*	99	48
Issatchenkia scutulata IFO 10069	*R*	93	50
Pichia heedii IFO 10020	*R*	81	24
Candida parapsilosis IFO 1396	*S*	98	60
Geotrichum candidum IFO 4601	*S*	88	78

4-hydroxy-2-butanone (4H2B) —microorganism→ optically active 1,3-butanediol (BDO)

Table 2
Screening of microorganisms producing optically active 1,3-BDO from the racemate

Microorganisms (yeasts, bacteria, fungi)
↓
Cultivation (10 ml / test tube, 30°C, 24-48h with shaking)
↓
Centrifugation and washing with saline
↓
Cells
↓
100mM KPB (pH 7.0) 2.5 ml
← Racemic 1,3-BDO 25mg
↓
Incubation (in test tube, 30°C, 48h with shaking)
↓
Centrifugation
↓
Supernatant (2ml) → Saturation with NaCl → Extraction with EtOAc (2m l) → Evaporation in *vacuo* → Acetylation with AcCl → HPLC Analysis, Determination of optical purity, Column: Chiralcel OB (Daicel)
↓
GLC Analysis
Determination of Residual 1,3-BDO
Column: Thermon 3000
Temp: 120°C

Strain	Absolute configuration	%*e.e.*	Residual 1,3-BDO yield (%)
Pseudomonas putida IFO 3738	*S*	75	50
Candida utilis IFO 0639	*S*	91	43
Candida inconspicua IFO 0621	*S*	97	50
Hansenula subpelliculosa IFO 0808	*S*	71	54
Kluyveromyces lactis IFO 1267	*S*	99	45
Pichia opuntiae IFO 10025	*S*	96	50
Fusarium solani IFO 5232	*S*	99	8
Ambrosiozyma philentoma IFO 1847	*S*	83	34
Talaromyces flavus IFO 7231	*S*	85	11
Candida parapsilosis IFO 1396	*R*	95	50
Candida intermedia IFO 0761	*R*	76	42
Candida maltosa IFO 1978	*R*	70	43
Aciculoconidium aculeatum IFO 10124	*R*	72	68
Geotrichum candidum IFO 4601	*R*	95	43
Lodderomyces elongisporus IFO 1676	*R*	72	49
Trichosporon cutaneum IFO 0743	*R*	69	48

racemic 1,3-BDO —microorganism→ optically active 1,3-BDO

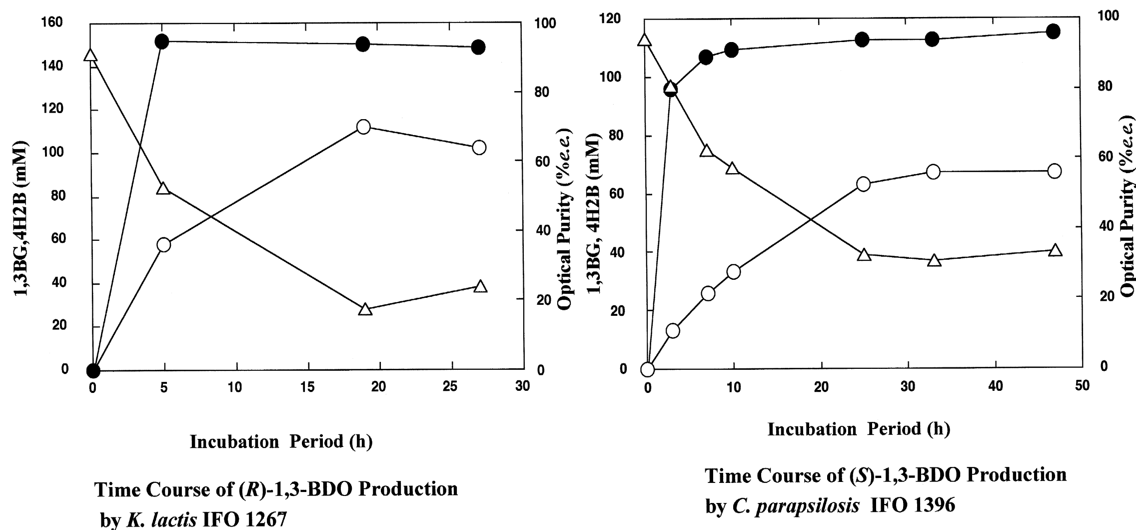


Fig. 1. Optically active 1,3-BDO production from 4H2B. (○) BDO; (△) 4H2B; (●) optical purity.

produced optically active 1,3-BDO from the racemate. High productivity and high optical purity were found in 16 strains. *C. parapsilosis* IFO 1396 produced (*R*)-1,3-BDO with 97% e.e., *K. lactis* IFO 1267 produced (*S*)-1,3-BDO with 99% e.e. from the racemate. [10] (Table 2).

4. Preparation of (*R*)- and (*S*)-1,3-BDO by the same strain or from the same material

The figures show the courses of production from 4H2B (Fig. 1) and from the racemate (Fig. 2) by *K. lactis* IFO 1267 and *C. parapsilosis* IFO 1396. *K.*

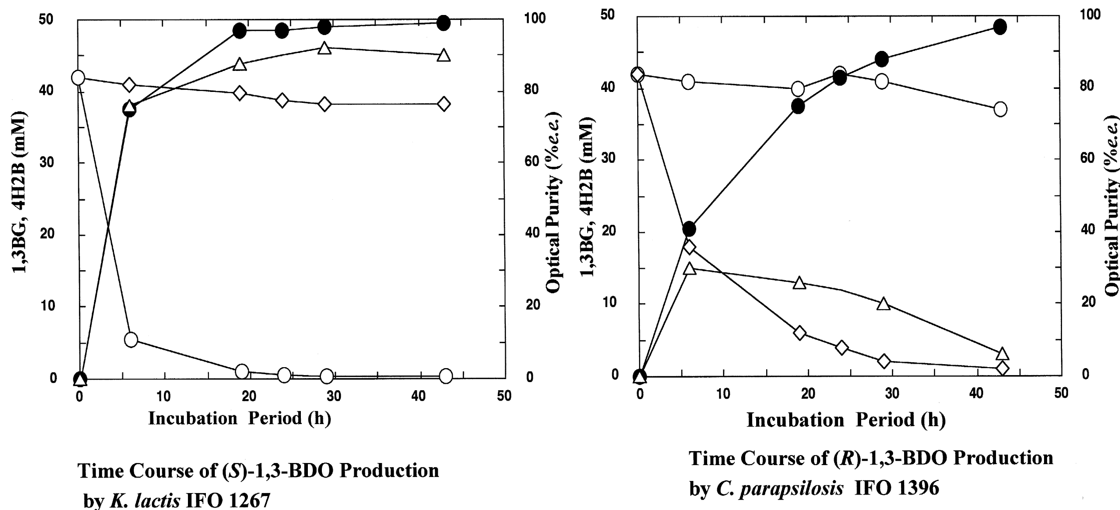
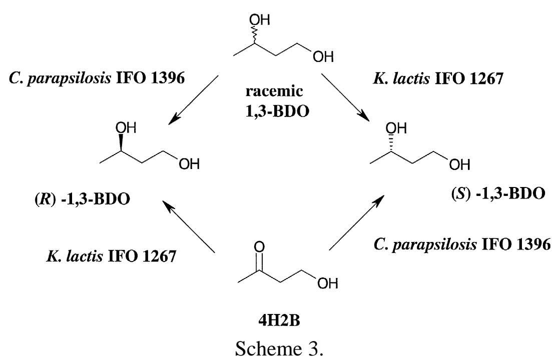


Fig. 2. Optically active 1,3-BDO production from the racemate. (○) (*R*)-1,3-BDO; (◇) (*S*)-1,3-BDO; (△) 4H2B; (●) optical purity.



Scheme 3.

lactis IFO 1267 produced (*R*)-1,3-BDO with 93% e.e. from 4H2B and (*S*)-1,3-BDO with 99% e.e. from the racemate. *C. parapsilosis* IFO 1396, on the contrary, produced (*S*)-1,3-BDO with 94% e.e. from 4H2B and (*R*)-1,3-BDO with 97% e.e. from the racemate. We indicated the methods of preparation of (*R*)-and (*S*)-1,3-BDO by the same strain or from the same material. (Scheme 3).

5. Large-scale preparation of (*R*)-1,3-BDO from the racemate by *C. parapsilosis* IFO 1396 [12]

Large-scale preparation of (*R*)-1,3-BDO from the racemate by *C. parapsilosis* IFO 1396 was done using a large fermentator with a working volume of 2000 l. The cells of 3000 l of YMBG (2.0% glucose, 0.5% Polypepton, 0.5% yeast extract, 0.3% malt extract, 1.0% racemic 1,3-BDO, pH 6.0) culture were harvested by centrifugation. Production of (*R*)-1,3-BDO was done in the reaction mixture containing 258 kg of the cells, 465 kg of water, 7.5 kg of calcium carbonate, and 20 kg racemic BDO. The temperature was maintain at 30°C. Agitation and

Table 4
Substrate specificity of (*S*)-BDH from *C. parapsilosis*
(*S*)-BDH: (*S*)-BDO Dehydrogenase.

Substrate	[S] (mM)	Rel. Act. (%)
Oxidation (NAD^+)		
(*S*)-1,3-BDO	50	100
($NADP^+$)	50	3.4
(*R*)-1,3-BDO	50	1.3
2-Propanol	100	337
(*RS*)-2-Butanol	100	245
(*S*)-2-Butanol	50	562
(*R*)-2-Butanol	50	18.8
(*RS*)-2-Pentanol	100	191
2,4-Pentanediol	100	240
(2*R*,4*R*)-2,4-Pentanediol	50	0.7
(*RS*)-2-Hexanol	50	156
(*S*)-2-Octanol	5	381
(*R*)-2-Octanol	5	0.0
(*S*)-1-Phenylethanol	50	502
(*R*)-1-Phenylethanol	50	6.4
Cyclohexanol	20	297
Methanol	100	1.0
Ethanol	100	5.4
Allyl alcohol	100	13.4
1-Propanol	100	8.5
1-Butanol	100	13.1
4-Hydroxy-2-butanone	100	11.1
1-Pentanol	100	6.8
2-Phenylethanol	100	0.0
Reduction (NADH)		
4-Hydroxy-2-butanone	100	100
(NADPH)	100	0.0
Acetone	100	299
2-Butanone	100	243
Acetophenone	20	296
Propionaldehyde	100	185

Table 3
Purification of (*S*)-1,3-BDO dehydrogenase

Step	Total activity (U)	Total proteins (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	7140	157,000	0.045	100
Protamine sulfate	6260	94,600	0.066	87.6
0–70% Ammonium sulfate	5460	78,700	0.069	76.5
Q-Sepharose FF	1730	8870	0.195	24.2
Phenyl-Toyopearl	969	191	5.07	13.6
Red-Sepharose	1100	22.1	49.6	15.3
Superdex 200	559	3.70	151	7.8
Mono Q	420	1.72	244	5.9

Table 5
Properties of (*S*)-BDH from *C. parapsilosis*

Reaction: (*S*)-1,3-BDO (OH, OH) + NAD^+ → 4-hydroxy-2-butanone (O, OH) + NADH + H^+

Specific activity	144 U / mg-protein (Tris-HCl, pH 9.0) at 30 ℃	
Molecular weight	Oligomer	140 K (GPC)
	Subunit	40 K (SDS-PAGE)
Optimal condition	Oxidation	50 ℃ (Tris-HCl, pH9.0)
		pH 9.0 (Tris-HCl)
	Reduction	pH 6.0 (KPB)
Substrate specificity	broad secondary alcohols > primary alcohols	
	(*S*)-1,3-BDO > (*R*)-1,3-BDO ($E = 62.1$)	
Inhibitors	SH-reagents, DTT, Hg^{2+} and *o*-PT	

Stereo-specific Secondary Alcohol Dehydrogenase

aeration were 66 rpm and 29 m^3/h. After reaction for 81 h (residual 1,3-BDO 41%), the reaction mixture was centrifuged to remove the cells, and condensed to 18.7 kg of slurry at 50°C in vacuo. After filtration, the solvent was removed by evaporation, and 3092 g of (*R*)-1,3-BDO (15.5% yield) was distilled in vacuo. Chemical purity was 98.8% and optical purity was 94.0 % e.e.

Table 6
Expression of CpSADH in *E. coli* JM109 (pKK-CpA1)

Induced expression
E.coli JM109 (pKK-CPA1)
↓ culture in 2xYT medium
↓ add IPTG to 1 mM at $OD_{600} = 1.0$
↓ culture at 30℃ for 8 h
(*S*)-BDH assay

Constitutive expression
E.coli JM109 (pKK-CPA1)
↓ culture in 2xYT medium
↓ culture at 30℃ for 16 h
(*S*)-BDH assay

(*S*)-BDH assay
Reaction mixture
50 mM Tris-HCl (pH 9.0)
50 mM (*S*)-1,3-BDO
2.5 mM NAD^+
enzyme
final 1 ml
↓ incubate at 30℃
read the absorbance at 340 nm
1U: 1 μ mol/min-NADH formation

2xYT medium (Bacto-Trypton, 20 g/l; Bacto-yeast extract,10g/l; Nacl, 10 g/l; ampicillin, 50 mg/l; pH 7.2)

	OD_{600}	(*S*)-BDH	
		U/ml-broth	U/mg-protein
C. parapsilosis	30.5	1.05	0.349
Induced expression	1.34	0.249	0.445
Constitutive expression	6.17	1.28	0.701

6. Purification and characterization of (S)-1,3-BDO dehydrogenase from *C. parapsilosis* IFO 1396 [13]

(S)-1,3-BDO oxidizing enzyme (CpSADH) was purified from *C. parapsilosis* IFO 1396. This enzyme could produce (R)-1,3-BDO from the racemate. The results of the enzyme purification are summarized in Table 3. The specific activity of the final preparation was about 5400-fold higher than that of the crude extract. The substrate specificity and properties of the enzyme are shown in Tables 4 and 5. As the enzyme oxidized (S)-1,3-BDO to 4H2B, the enzyme was thought to oxidize the hydroxy residue on the 3-position but not the 1-position. From these results, the enzyme was found to be a secondary alcohol dehydrogenase. Previously, secondary alcohol dehydrogenase specific for (R)-2-alcohols, such as (R)-2-butanol, were reported from methylotrophic bacteria and yeasts, such as *Pseu-*

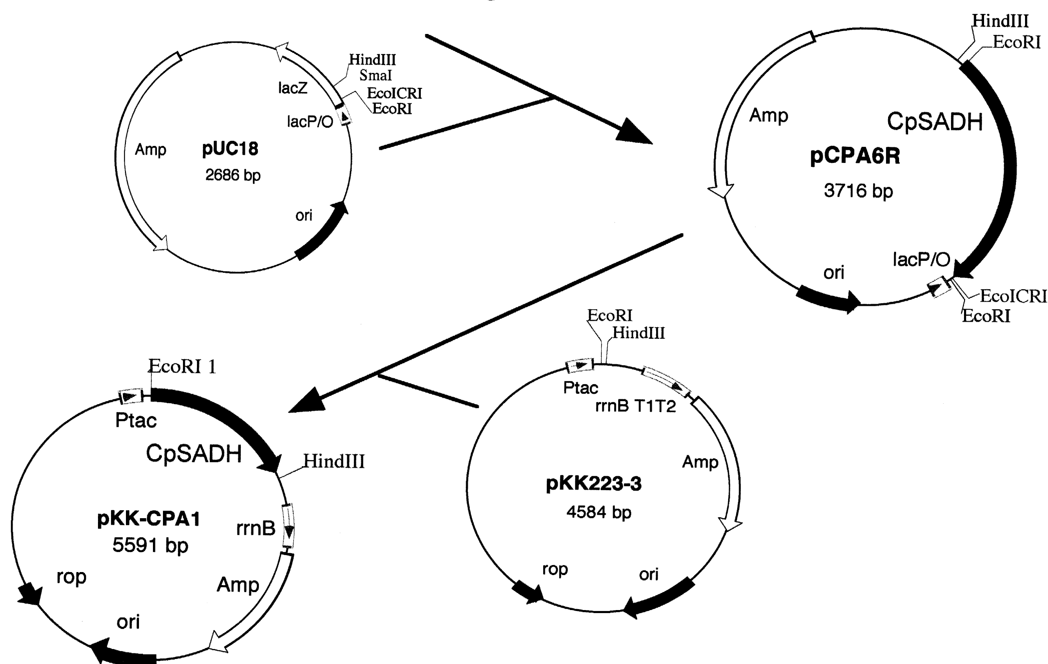


Fig. 3. Construction of CpSADH expression plasmid.

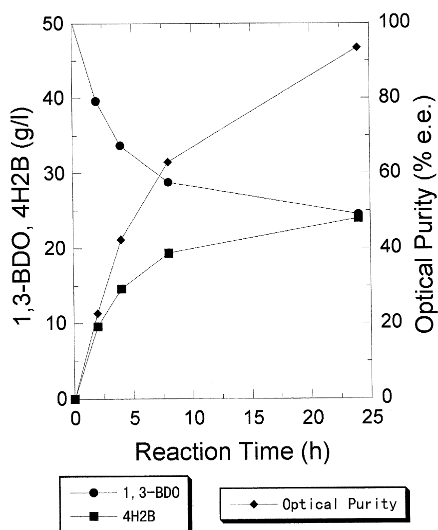
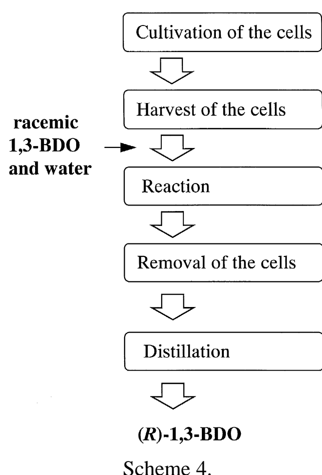
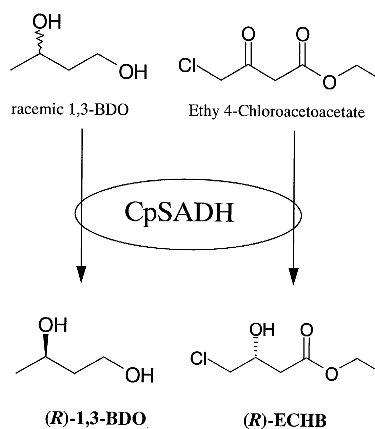


Fig. 4. Preparation of (R)-1,3-BDO by a recombinant *E. coli* expressing CpSADH.



Scheme 4.

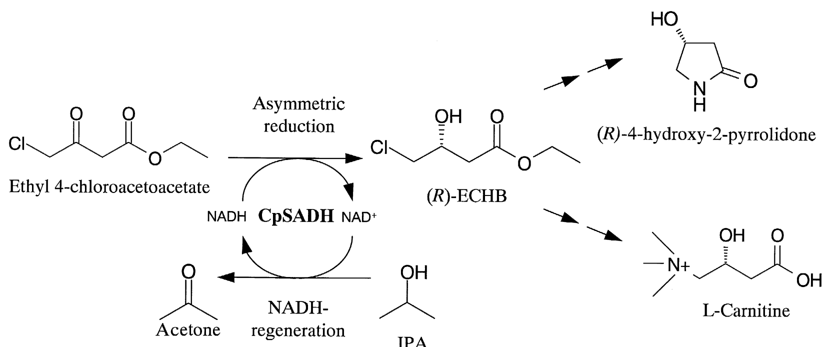


Scheme 6.

domonas [14] and *Pichia* [15], but a secondary alcohol dehydrogenase specific for (S)-2-alcohols has not been reported.

7. Cloning and expression of a gene coding for a secondary alcohol dehydrogenase from *C. parapsilosis* IFO 1396 in *Escherichia coli*

A gene encoding CpSADH that catalyzed the oxidation of (S)-1,3-BDO to 4H2B was cloned from *C. parapsilosis*. This CpSADH-gene consisted of 1009 nucleotides coding for a protein with M_r 35,964. The overall amino acid sequence identity with alcohol dehydrogenases from *Saccharomyces cerevisiae* (ScADH1, ScADH2, ScADH3, and ScADH5), alcohol dehydrogenase I from *Zymomonas mobilis* (ZmADH1), and the NADP-dependent secondary alcohol dehydrogenase from



Scheme 5.

Thermoanaerobium brockii (TbADH) were estimated to be 31.3%, 30.5%, 28.0%, 29.9%, 31.3%, and 23.9% identity, respectively. The CpSADH activity of a recombinant *E. coli* strain was more than two times higher than that of *C. parapsilosis* (Table 6). A recombinant *E. coli* JM 109 strain harboring the expression plasmid, pKK-CAP1 (Fig. 3), produced (*R*)-1,3-BDO (93.5% e.e., 94.7% yield) from the racemate (5% 1,3-BDO) at 30°C without any to regenerate NAD^+ from NADH. [16] (Fig. 4).

Our industrial "Green" bio-process of (*R*)-1,3-BDO is shown in Scheme 4.

8. Preparation of ethyl (*R*)-4-chloro-3-hydroxy-butanoate (ECHB) by the recombinant *E. coli* expressing CpSADH

The recombinant *E. coli* expressing CpSADH produced ethyl (*R*)-4-chloro-3-hydroxy-butanoate from ethyl 4-chloro-3-oxo-butanoate (4%) with isopropanol (IPA, 2.8%) [17] (Scheme 5). Its maximum yield (36.6 g/l, 99% e.e.) was reached after 14 h of incubation at 15°C. This asymmetric reduction system didn't require an additional NADH-regeneration system such as a glycolytic pathway and glucose dehydrogenase with corresponding substrate. CpSADH served as both the synthetic (asymmetric reduction) and regenerating (NADH-regeneration) enzyme. Isopropanol was oxidized in order to regenerate NADH.

9. Conclusion and outlook

We have demonstrated that the enantio-selective oxidation and asymmetric reduction systems using the recombinant *E. coli* expressing CpSADH are efficient and convenient systems to synthesize many chiral alcohols, for instance, (*R*)-1,3-BDO and (*R*)-ECHB on an industrial scale. (Scheme 6).

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