Gene Cloning and Expression of Leifsonia Alcohol Dehydrogenase (LSADH) Involved in Asymmetric Hydrogen-Transfer Bioreduction to Produce (R)-Form Chiral Alcohols

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The gene encoding Leifsonia alcohol dehydrogenase (LSADH), a useful biocatalyst for producing (R)-chiral alcohols, was cloned from the genomic DNA of Leifsonia sp. S749. The gene contained an opening reading frame consisting of 756 nucleotides corresponding to 251 amino acid residues. The subunit molecular weight was calculated to be 24,999, which was consistent with that determined by polyacrylamide gel electrophoresis. The enzyme was expressed in recombinant Escherichia coli cells and purified to homogeneity by three column chromatographies. The predicted amino acid sequence displayed 30–50% homology to known short chain alcohol dehydrogenase/reductases (SDRs); moreover, the NADH-binding site and the three catalytic residues in SDRs were conserved. The recombinant E. coli cells which overexpressed lsadh produced (R)-form chiral alcohols from ketones using 2-propanol as a hydrogen donor with the highest level of productivity ever reported and enantioemic excess (e.e.).

Key words: alcohol dehydrogenase; Leifsonia sp.; asymmetric bioreduction; biocatalyst; (R)-chiral alcohols

In recent years, enantiometrically pure compounds, including D- and L-amino acids, organic acids, amines, alcohols, epoxides, and so on, have been produced with the use of biocatalysts, because such methods are superior to general chemical methods in terms of enantioselectivity. Among them, secondary alcohols are the most important chiral syntons for pharmaceuticals and agrochemicals.

Noyori et al. have developed efficient transfer hydrogenation catalysts: ruthenium complexes of BINAP and derivatives, with which not only hydrogen but also formic acid or 2-propanol serves as the hydrogen donor for ketone reduction. But, in many cases, difficulties remain in the cost of the catalyst and the operability, and in attaining sufficient optical purity and productivity. An alternative to chemical asymmetric reduction processes is a biocatalytic transformation system using enzymes or microorganisms.

Enzymatic acylations for the resolution of chiral alcohols were developed in the 1980s, and several kinds of lipases have been used as biocatalysts, but, in most cases, for enzymatic resolution, only a 50% yield of desired intermediate is obtained.

Many examples of the reduction of ketones with reductases and dehydrogenases have also been described, because such processes theoretically produce alcohol with 100% conversion from ketone. But, in most cases, they have the disadvantages of a narrow substrate spectrum and low productivity due to the need for a cofactor regeneration system such as NADH and NADPH, although they show rather high enantioselectivity. Therefore, such bioreduction processes are economical only when the cofactor can be regenerated in situ in a second catalytic cycle, for example, formate/formate dehydrogenase (FDH) or glucose/glucose dehydrogenase (GDH). More recent studies concerning biosymmetric reduction have revealed that much enhanced productivity can be obtained only when a powerful cofactor regenerating system is coupled with the process.

From the viewpoint of the regeneration of NAD(P)H, 2-propanol is another suitable hydrogen donor for bioreduction because of its chemical properties and low cost. Recently, Itoh et al. reported that phenylacetaldehyde reductase (PAR) from the styrene-assimilating Rhodococcus (former Corynebacterium) sp. strain ST-10 is a unique NADH-dependent alcohol dehydrogenase (ADH) with a broad substrate range and high enantioselectivity to give (S)-form alcohols without an additional coenzyme regeneration system, because the enzyme itself is able to regenerate NADH in the presence of 2-propanol. Therefore, a recombinant PAR or its mutated enzyme (Sar268) system and chemotolerant ADH reported in the R. ruber DSM44541 strain, are regarded as superior asymmetric hydrogen-transfer biocatalysts with which to produce (S)-alcohols.

We have also found a novel ADH (LSADH) producing (R)-alcohols in Leifsonia sp. strain S749, and have...
characterized it in detail.\textsuperscript{17,18} LSADH is a NAD\textsuperscript{+}-dependent ADH, and it produces many chiral alcohols from ketones, and $\alpha$-keto and $\beta$-ketoesters with high enantioselectivity. This process can function without the additional coenzyme regeneration system in the presence of 2-propanol in a similar manner to PAR (Fig. 1).\textsuperscript{14,15,17}

In this report, we describe the cloning, sequence analysis, and expression in \textit{Escherichia coli} of the gene encoding LSADH from \textit{Leifsonia} sp. S749, and the purification of the recombinant enzyme. We have also confirmed that the established expression system functions well as a hydrogen-transferring biocatalyst from 2-propanol to produce chiral (R)-alcohols.

### Materials and Methods

**Chemicals.** SDS–PAGE molecular weight standards (low range) were purchased from Nippon Bio-Rad Laboratories (Tokyo). The marker protein kit for HPLC analysis, and expression in \textit{Escherichia coli} were kindly supplied by Sumitomo Chemical (Osaka, Japan). All other chemicals used in this study were of analytical grade and were commercially available.

**Bacterial strains, vectors, and culture conditions.** \textit{Leifsonia} sp. strain S749,\textsuperscript{17} a coryneform Gram positive bacterium, was used as the source of chromosomal DNA. \textit{E. coli} XL1-Blue MRF\textsuperscript{+} (\textit{Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F proAB lacIQZD M15 (Tetr)]}) was used as a host strain for the construction of a cosmid genomic library. \textit{E. coli} JM109 (\textit{recA1, endA1, gyrA96, thi, hsdR17}(rK\textsuperscript{−} mK\textsuperscript{−})), e14\textsuperscript{−} (mcrA\textsuperscript{−}), supE44, relA1, \textit{Δ(lac-proAB)/F[traD36, proAB\textsuperscript{+}, lac I, lacZΔM15]), BL21(DE3) (F\textsuperscript{−} ompT, hsdS\textsubscript{B}(rK\textsuperscript{−} mK\textsuperscript{−})\textsuperscript{gal}, lacI1 857, ind1, Sam7, nin5, lacUV5-T7gene1, dcM(DE3)), and BL21 (F\textsuperscript{−} ompT hsdS\textsubscript{B}(rK\textsuperscript{−} mK\textsuperscript{−}) gal dcM) were used as host strains for the expression of LSADH. Cosmid pWE15 (Toyobo, Osaka) was used as a vector for the construction of the genomic library. Plasmids pGEM-T easy vector (Promega, Madison, WI), pUC118 and pGEM5Zf(+) (Promega) were used as cloning vectors. Plasmids pET21b (Novagen/Merck Biosciences, San Diego, CA), pKK223-3 and pTrc99A (Amersham Pharmacia Biotech, Tokyo) were also used as expression vectors. For gene cloning and enzyme purification, \textit{E. coli} cells were cultivated at 37°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.0) containing 0.1 mg/ml ampicillin, unless otherwise noted. For induction of the gene under the control of the lac promoter, 0.4 mM isopropyl $\beta$-D-thiogalactoside (IPTG) was added to LB medium. To select recombinant cells harboring pGEM-T easy or pUC118 with inserted DNA, 0.01% (w/v) 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside (X-Gal) was added to the 1.5% (w/v) LB agar. For the preparation of genomic DNA from \textit{Leifsonia} sp. strain S749, the bacteria were cultivated at 30°C for about 18h in a liquid medium containing 0.5% (w/v) peptone and 0.5% (w/v) yeast extract (pH 7.0).

**Partial NH$_2$-terminal and internal amino acid sequences of LSADH.** The enzyme was electrophoresed on an SDS–PAGE gel, transferred to a PVDF (polyvinylidene difluoride) membrane (Bio-Rad) using a semidry electroblotting apparatus (NA-1512, Nippon Eido, Tokyo) at constant current of 0.8 mA/cm\textsuperscript{2} gel for 90 min by the method of Hirano and Watanabe,\textsuperscript{19} and then stained with Coomassie brilliant blue G-250. The NH$_2$-terminal amino acid sequence of the enzyme on the PVDF membrane was determined using a HP G1005A protein sequencing system (Hewlett Packard, Palo Alto, CA), and its internal amino-acid was determined by APRO Life Science Institute (Tokushima, Japan).

**General recombinant DNA techniques.** The genomic DNA from \textit{Leifsonia} sp. strain S749 was isolated as described by Hopwood et al.\textsuperscript{20} Plasmid DNA was purified with a kit from Qiagen (Chatsworth, CA). Restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan), Toyobo Biochemicals (Osaka, Japan), and New England Biolabs (Herts, UK) and used according to the manufacturer’s directions.
ligase was purchased from New England BioLabs. DNA polymerases (Takara Ex Taq and Takara LA Taq) were purchased from Takara Shuzo. Transformation of *E. coli* with plasmid DNA by electroporation was performed under standard conditions with a Gene Pulser electroporation system (Bio-Rad). DNA hybridization was non-radioactively performed with an AlkPhos direct labelling and detection system using a CDP-Star kit (Amersham Biosciences, Tokyo, Japan) following the manufacturer’s instructions. Other general DNA manipulation procedures were carried out as described by Sambrook et al.21) All the primers were purchased from Espec Oligo Service (Ibaraki, Japan).

**Preparation of the probe for colony hybridization.** Two degenerate oligonucleotide primers were synthesized for cloning the *lsadh* gene based on the NH2-terminal amino acid sequence of native LSADH (AQYDVADRSAIVTG) described previously17) and tryptic digests of LSADH (IAVNNAGIGGGEA), respectively: 5’-primer, 5’-CARTAYGAYGTCNGAHMG-3’, and 3’-primer, 5’-CCDATICNCGRTRTNNAC-3’. Degenerate positions are indicated by R for A or G, Y for T or C, M for A or C, H for A, T, or C, N for all bases, and I for inosine. PCR amplification was carried out in a reaction mixture of 20 µl containing 100 pmol of each of the two primers, 90 ng of *Leifsonia* sp. strain S749 chromosomal DNA, 0.25 mM of each deoxynucleotide, and 2.5 units of *E. coli* extract (Stratagene, La Jolla, CA), and used to transfect phage λ particles with Gigapack III gold packaging extract (Stratagene, La Jolla, CA), and used to transfect *E. coli* XL1-Blue MRF’ cells to construct a genomic library. The library was screened by colony hybridization with the alkaline phosphatase-labeled 280-bp DNA fragment. The colony hybridization was performed following the manufacturer’s directions. A homology-based search was performed with the FASTA program.

**Construction of the vector for expressing *lsadh* in *E. coli*.** Primers used for the amplification of genes by PCR are listed in Table 1. Six recombinant plasmid vectors were constructed as follows: The primers ULA-F and ULA-R were used to amplify the 0.8-kb *lsadh* fragment from the genomic DNA of *Leifsonia* sp. S749 (pWELA6), and the PCR fragment purified on agarose gel was inserted into the BamHI-PstI site of vector pUC118 to give pULA. In a similar manner, primers KLA-F and ULA-R were used to amplify *lsadh* of vector pKK223-3 to give pKLA. The *lsadh* gene amplified using primers TLA-F and ULA-R was purified and then inserted into the Ncol-PstI site of vector pKELA. pKELA was also introduced into *E. coli* BL21. pULA was introduced into *E. coli* JM109. pKLA was also introduced into *E. coli* BL21(DE3). pKELA was also introduced into *E. coli* JM109 XL1Blue MRF’ and BL21.

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**Table 1. Primers Used for PCR Amplification**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequencea</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>ULA-F</td>
<td>5’T-GTCGGATCCCTGAAGGAGATTTTCATG3’</td>
<td>BamHI</td>
</tr>
<tr>
<td>ULA-R</td>
<td>5’T-GGCGCTGACCGGGGTCAG-3’</td>
<td>PstI</td>
</tr>
<tr>
<td>KLA-F</td>
<td>5’T-GAAGGAGGAATTGCTCGTACGAC-3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>TLA-F</td>
<td>5’T-GGACATTCTACGCTCAGTACGAC-3’</td>
<td>NcoI</td>
</tr>
<tr>
<td>ELA-F</td>
<td>5’T-GGGATCCATGACCTCGTACGAC-3’</td>
<td>Ndel</td>
</tr>
</tbody>
</table>

*aUnderlined nucleotides represent the restriction sites.*
Cloning of *Leifsonia* ADH Gene for Asymmetric Bioreduction

Purification of recombinant LSADH. All purification procedures were performed at 0–4 °C in 20 mm KPB (pH 7.0) containing 10% (v/v) glycerol, unless otherwise specified. *E. coli* BL21 (pKELA) cells were grown with shaking at 37 °C for 18 h in LB medium containing 100 μg/ml of ampicillin. The washed cells (1.17 g wet weight), collected from 100 ml of culture broth, were suspended in 20 ml of the buffer and then disrupted with an ultrasonic oscillator (Insonator 201 M, Kubota, Osaka, Japan) for 5 min. After centrifugation (13,000 × g, 30 min), the supernatant was applied to a DEAE-Toyopearl 650 M (Tosoh, Tokyo) column (2.5 by 13 cm) equilibrated with the buffer. The enzyme was eluted with a linear 0 to 0.6 M NaCl gradient in the same buffer. The fractions with strong enzyme activity were collected (total volume, 28 ml). The solution, mixed with ammonium sulfate up to a concentration of 0.6 M, was applied to a Butyl-Toyopearl 650 M (Tosoh) column (2.5 by 13 cm) which had been equilibrated with the buffer containing 0.6 M ammonium sulfate. The enzyme was eluted with a linear 0 to 0 M ammonium sulfate gradient in the same buffer. The fractions with strong enzyme activity were collected and concentrated using a Centriprep YM-30 (cutoff MW, 30,000, Millipore, Billerica, MA), and stored as a purified enzyme preparation at −20 °C.

Enzyme and protein assays. Activity was assayed spectrophotometrically at 25 °C by measuring the decrease in absorption at 340 nm of NADH, as described in a previous paper using trifluoroacetophenone (PTK) as a substrate. One unit of enzyme was defined as the amount that converted 1 μmol of NADH and PTK in 1 min. The protein concentration was estimated using a Bio-Rad protein assay kit with bovine serum as a standard protein. SDS–PAGE was performed in a 14.0% polyacrylamide slab gel with the Tris-glycine buffer system. The molecular mass of the enzyme subunit was determined from the relative mobility of standard proteins.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper is available from the DDBJ, EMBL, and GenBank databases under accession no. AB213459.

Production of chiral alcohols coupled to NADH regeneration with 2-propanol. To determine the enantiomeric purity of some products and assess the coupling system of NADH regeneration, we constructed a system with LSADH and 2-propanol (Fig. 1). The reaction mixture used for measuring the products from ketones consisted of 1 μmol NAD⁺, 100 μmol KPB (pH 7.0), 10% (v/v) 2-propanol, *E. coli* cells from 1 ml of culture broth containing approximately 5 units of LSADH, and one hundred mg of each substrate (10% w/v) in a total volume of 1 ml, was suspended in a 2-ml polypropylene tube in a BioShaker MBR-022 (Taitec, Saitama, Japan) with shaking (2,500 rpm) for 24 h at 25 °C. After the reaction, the mixture was extracted twice with ethyl acetate. The combined ethyl acetate extracts were dried with Na₂SO₄, and analyzed by gas chromatography (GC) or high performance liquid chromatography (HPLC), as described previously. GC was performed using a Shimadzu GC-18A system (Kyoto, Japan) equipped with a capillary column (J & W capillary column DB-1, 0.25 mm × 30 m, Agilent Technologies, Palo Alto, CA) with an FID (flame ionization detector) under the following conditions: injection and detection temperature of 250 °C, column temperature of 40 °C for 5 min, raised to 100 °C at 5 °C/min, the split ratio was 40, and a flow rate of 1 ml/min of He. The products and substrates showed the following retention times (min): 2-heptanol, 6.3; 2-heptanol, 7.0; ethyl 3-methyl-2-oxobutanoate, 12.1; ethyl 3-methyl-2-hydroxybutanoate, 12.9. An injection and detection temperature was 250 °C, and column temperature of 70 °C for 5 min was raised to 250 °C at 5 °C/min, and a flow rate of 1 ml/min of He. Ethyl 4-chloro-3-oxobutanoate showed the retention time of 11.1 min and ethyl 4-chloro-3-hydroxybutanoate of 12.3 min. GC was also performed using a Shimadzu GC-14 A system equipped with a coiled column (3 mm × 2 m) packed with Thermon 1000 (5% on Chromosorb W) with an FID, column temperature of 70 °C, injection and detection temperatures of 200 °C, and a flow rate of 50 ml/min of N₂. The product and substrates showed the following retention times (min): ethyl 3-oxobutanoate, 7.6; ethyl 3-hydroxybutanoate, 10.1.

To determine the absolute configuration of certain alcohols, the product was analyzed using GC (HP 6890 GC system, Agilent Technologies) with a Chrompack CP-cyclodextrin-β-236-N19 chiral column (0.25 mm by 25 m, 0.25 μm film, Varian, Palo Alto, CA) and a FID. Helium gas was used as a carrier at 15 psi (0.5 ml/min), the split ratio was 50, and the injection and detection temperatures were 250 °C. The column temperature was maintained isothermally at 80 °C. The products and substrates showed the following retention times (min): ethyl pyruvate, 3.1; ethyl (R)-lactate, 4.0; ethyl (S)-lactate, 4.2. When the column temperature was maintained at 130 °C, PTK, (S)-PTE, and (R)-PTE indicated the following retention times (min): PTK, 1.8; (S)-PTE, 6.7; (R)-PTE, 7.3. When the column temperature was kept at 160 °C, 2,3′-dichloroacetophenone, (S)-2-chloro-1-(3-chlorophenyl)ethanol, and its (R)-form indicated the following retention times (min): 2,3′-dichloroacetophenone 8.5; (S)-2-chloro-1-(3-chlorophenyl)ethanol, 12.7; (R)-2-chloro-1-(3-chlorophenyl)ethanol 13.1.
similar to those utilized in codon, ATG, and ending with a termination codon, lsadh was subcloned and sequenced. Nucleotide sequence cosmids, pWELA6, was analyzed by Southern hybridization. One of the positive recombinant Results (Table 2), 0.5 ml/min, 254 nm and 30°C with the retention times of 7.2 min for the (R)-derivative and 7.4 min for the (S)-derivative of 2-heptanol; (OB-H, hexane/2-propanol (9:1), 1.0 ml/min, 220 nm and 30°C with the retention times of 11.6 min for ethyl (S)-3-hydroxybutanoate and 12.6 min for (R)-form; (OB-H, hexane/2-propanol (9:1), 1.0 ml/min, 220 nm and 30°C with the retention times of 7.4 min for ethyl (R)-4-chloro-3-hydroxybutanoate and 7.9 min for (S)-form; (OD-H, hexane/2-propanol (9:1), 0.5 ml/min, 220 nm and 30°C with the retention times of 7.9 min for ethyl (S)-2-hydroxy-3-methylbutanoate and 8.4 min for (R)-form.

**Results**

**Sequence analysis of lsadh**

Total DNA isolated from *Leifsonia* sp. S749 cells was partially digested with Sau3AI into fragments of 15 to 20 kb, which were then inserted into pWE15. To screen the cosmids genomic library thus obtained, a probe DNA was synthesized by PCR using primers designed for the cosmid genomic library thus obtained, a probe DNA was synthesized by PCR using primers designed for the following names and properties in the order (designated name, promoter, origin of the SD sequence). The restriction site of the 3′-terminal region of lsadh in each vector was PstI for pULA, pKLA, pTLA, pUEL A, and pKELA, and PstI and NotI for pELA.

A search of protein amino acid sequence databases (DBJ, GenBank, EMBL and PIR) revealed that LSADH was similar to the SDR family enzymes (identity %, viz., cyclohexanone dehydrogenase from *Acinetobacter* sp. (50.6), 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase from *Sphingomonas pau cimobilis* (42.8), 4-arabinitol 2-dehydrogenase from *Pichia stipitis* (27.9), glucose 5-dehydrogenase from *Glucobacter suboxydans* (31.9), 3-oxoacyl-[acyl-carrier-protein] reductase from *E. coli* (37.7), glucose 1-dehydrogenase from *Bacillus megaterium* (32.7), and acetocacetyl-CoA reductase from *Zoogloea ramigera* (35.3). The predicted amino acid sequence of LSADH displayed 30–50% homology to known SDRs, including the putative proteins which had been subscribed with SDR. The amino acid sequence alignment and accession numbers are shown in Fig. 2.

**Construction of E. coli transformants overexpressing LSADH**

To overexpress *lsadh* in *E. coli* cells, the six expression vectors in Table 2 were constructed. They had the following names and properties in the order (designated name, promoter, origin of the SD sequence and downstream region, original vector): vector pULA, lac, AGGGA from *Leifsonia* sp. S749 chromosomal DNA, pUC118; vector pKLA, tac, AGGA from pKK223-3, pKK223-3; vector pTLA, trc, AGGA from pTrc99A, pTrc99A; vector pELA, T7, AGGA from pET21b, pET21b; vector pUEL A, lac, AGGA from pET21b, pUC118; vector pKELA, tac, AGGA from pET21b, pKK223-3. *E. coli* BL21 carrying pKELA showed the highest level of enzyme activity (5.2 units/ml culture broth) without induction by IPTG. It was 170-fold higher than that for *Leifsonia* sp. S749 (Table 3). The *lsadh* gene was so highly expressed in *E. coli* cells
that more than 50% of the total cellular protein consisted of LSADH, as shown in lane 2 of Fig. 3.

**Purification of the recombinant enzyme and its molecular mass**

Purification was performed using a buffer containing 10% glycerol, which was effective in preventing the aggregation of LSADH proteins and loss of activity. From the result in Table 4, the amount of LSADH was estimated to be 58% of the total amount of soluble protein in the cell-free extract. According to analytical HPLC on TSK gel G3000SWXL, the molecular mass of the purified recombinant enzyme was estimated to be 105 kDa. SDS–PAGE revealed a single band, and the subunit molecular mass was 26 kDa. These results show that LSADH is a homotetramer protein, the same as the native LSADH purified from *Leifsonia* sp. S749.17

![Amino Acid Sequence Alignment of LSADH](image-url)

Production of chiral alcohols by E. coli overexpressing LSADH coupled with in situ regeneration of NADH

The optimal reaction conditions observed for the free enzyme reaction (1 mM NAD\(^+\), 10% v/v 2-propanol, pH 7.0)\(^{18}\) were adopted for the E. coli biocatalyst system. As exemplified in Table 5, enantioselective reduction of the ketones and keto esters by this process using LSADH with 2-propanol as a hydrogen donor gave chiral alcohols with an enantiomeric purity of > 99% e.e. Moreover, conversions of five substrates were almost 100% (100 mg product/ml reaction mixture: 0.53–0.88 M) except for 2-heptanone and ethyl pyruvate under conditions without optimization of the reaction for each substrate. The relatively low conversions for (R)-2-heptanol and ethyl (R)-lactate were probably due to the equilibrium of the reaction, which have been improved by the addition of a higher concentration of 2-propanol (data not shown). The results clearly demonstrate the ability of intact E. coli cells to express \(lsadh\) as an asymmetric bioreduction biocatalyst for producing (R)-form chiral alcohols.

Discussion

In order to establish a suitable expression system of LSADH and also to study its structural and functional relationships, we cloned the \(lsadh\) gene, determined the primary structure of the enzyme, and expressed the \(lsadh\) gene in E. coli cells. A homology-based search of the deduced amino acid sequence of the LSADH showed that it is similar to the short-chain alcohol dehydrogenase/reductase (SDR) family. A possible consensus sequence of the coenzyme-binding site of the SDR family enzyme, GXXXGXG (amino acid positions 15 to 21 of LSADH) and the amino acid residues reported to be important for activity among the SDR family enzymes, Ser 152, Tyr165, and Lys169,\(^{25}\) are fully conserved in the \(lsadh\) sequence. The existence of the coenzyme-interacting Asp residue (position 42), which regulates specificity for NADH or NADPH, agrees with the coenzyme specificity of this enzyme for NADH. Thus the sequence data clearly indicates that LSADH of Leifsonia sp. belongs to the SDR family. However, in spite of the homology in primary structure, LSADH is quite different in substrate specificity from the dehydrogenases described in Fig. 2\(^{26-29}\) and from other (R)-form alcohol-producing ADHs previously reported.\(^7\)

Table 3. LSADH Activity in Cell-Free Extracts of E. coli Transforms

<table>
<thead>
<tr>
<th>Host</th>
<th>Plasmid</th>
<th>Activity (U/ml of culture)(^a)</th>
<th>With IPTG</th>
<th>Without IPTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>pULA</td>
<td>0.03</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>pKLA</td>
<td>0.04</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>pTLA</td>
<td>0.04</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>pELA</td>
<td>0.81</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>pUELA</td>
<td>0.59</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>XL1BlueMRF</td>
<td>pKELA</td>
<td>1.5</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>BL21</td>
<td>pKELA</td>
<td>2.3</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Activity was measured using PTK as a substrate.

Table 4. Summary of the Purification of LSADH from E. coli BL21 (pKELA)

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification factor (fold)</th>
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<tr>
<td>Cell extract</td>
<td>84.5</td>
<td>518</td>
<td>6.1</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>55.0</td>
<td>461</td>
<td>8.4</td>
<td>89.0</td>
<td>1.37</td>
</tr>
<tr>
<td>Butyl-Toyopearl</td>
<td>31.9</td>
<td>332</td>
<td>10.4</td>
<td>64.1</td>
<td>1.70</td>
</tr>
</tbody>
</table>

Fig. 3. Purification of Recombinant LSADH from E. coli (pKELA).

Lane 1, molecular weight standards, including (from top to bottom) phosphorylase B (Mr, 97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400); lane 2, cell extract (5 μg); lane 3, DEAE-Toyopearl (5 μg); lane 4, Butyl-Toyopearl (5 μg). The gel was stained with Quick-CBB (Wako Pure Chemicals, Osaka, Japan).
ATG initial codon, greatly influenced the expression level of LSADH.

As shown in Table 5, we found that an E. coli biocatalyst expressing LSADH can regenerate NADH in the presence of 2-propanol to produce chiral alcohols with a high enantioselectivity and yield. The conversions of PTK, ethyl 3-methyl-2-oxobutanoate, ethyl 3-oxobutanoate, ethyl 4-chloro-3-oxobutanoate and 2,3-dichloroacetophenone were almost 100% (100 mg/ml reaction mixture) without optimization for each substrate after 24 h of reaction, suggesting further productivity. In fact, we have confirmed that production of ethyl (S)-4-chloro-3-hydroxybutanoate, a useful building block of HMG-CoA reductase inhibitor, reached a level of more than 350 g/l reaction mixture (data will be reported elsewhere), which is comparable with the productivity reported by Shimizu et al. using recombinant E. coli biocatalyst expressing NADPH-dependent carbonyl reductase from Candida magnoliae and glucose dehydrogenase.9) Our bioprocess is superior to the previous ones in the following points: (1) a simple reaction system because there is no need for additional NADH-regenerating system, (2) easy handling in downstream processing and low cost due to using 2-propanol as a hydrogen donor and coexisting solvent, (3) application to versatile ketones because of LSADH’s broad substrate range and high enantioselectivity.17,18)

The rather low conversions observed for (R)-2-heptanol and ethyl (R)-lactate are considered to be due to the equilibrium of the reaction. For example, in the case of 2-heptanol, the $K_{eq}$ value of [2-heptanol]•[NAD$^+$]/[2-heptanone][NADH][H$^+$] is $4.0 \times 10^{-8}$ M,13) and that of [2-propanol][NAD$^+$]/acetone][NADH]-[H$^+$] is $8.0 \times 10^{-7}$ M. Therefore, the theoretical conversion of the product was calculated to be 81% under the tested conditions from the calculation. A quite similar conversion (78%) was in fact observed when the same reaction was carried out using free LSADH,18) suggesting that 2-propanol in the E. coli cells was lowered by the barrier of the cell membrane of E. coli. This drawback can be overcome by optimization of the reaction and process engineering, such as the addition of a higher concentration of 2-propanol, elimination of the acetone produced from the reaction mixture, and an organic solvent and water two-phase reaction system.9,10) Thus the established expression system of LSADH in E. coli should provide a powerful means to obtain optically pure alcohols for industry.

Acknowledgments

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Table 5. Conversion and Enantioselectivity of the E. coli (pKELA) Biocatalyst for the Reduction of Ketones

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)*</th>
<th>Product</th>
<th>Enantiomeric excess (%)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>O</strong>229(OH)**</td>
<td>229</td>
<td>(R)-form</td>
<td>99</td>
<td>57</td>
</tr>
<tr>
<td><strong>O</strong>488(OH)**</td>
<td>488</td>
<td>(R)-form</td>
<td>&gt; 99</td>
<td>58</td>
</tr>
<tr>
<td><strong>O</strong>33(OH)**</td>
<td>33</td>
<td>(R)-form</td>
<td>&gt; 99</td>
<td>100</td>
</tr>
<tr>
<td><strong>O</strong>309(OH)**</td>
<td>309</td>
<td>(R)-form</td>
<td>&gt; 99</td>
<td>100</td>
</tr>
<tr>
<td><strong>O</strong>809(OH)**</td>
<td>809</td>
<td>(S)-form</td>
<td>&gt; 99</td>
<td>100</td>
</tr>
<tr>
<td><strong>O</strong>100(CF3OH)**</td>
<td>100</td>
<td>(S)-form</td>
<td>&gt; 99</td>
<td>100</td>
</tr>
<tr>
<td><strong>O</strong>67(CF3ClOH)**</td>
<td>67</td>
<td>(S)-form</td>
<td>&gt; 99</td>
<td>98</td>
</tr>
</tbody>
</table>

*Activity was measured by NADH consumption and is displayed when that for PTK is 100%.
References


