Purification and Characterization of a Novel Alcohol Dehydrogenase from *Leifsonia* sp. Strain S749: a Promising Biocatalyst for an Asymmetric Hydrogen Transfer Bioreduction

Kousuke Inoue, Yoshihide Makino, and Nobuya Itoh*

Biotechnology Research Center, Toyama Prefectural University, Kosugi, Toyama, Japan

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To find microorganisms that could reduce phenyl trifluoromethyl ketone (PTK) to (S)-1-phenyltrifluoroethanol [(S)-PTE], styrene-assimilating bacteria (ca. 900 strains) isolated from soil samples were screened. We found that *Leifsonia* sp. strain S749 was the most suitable strain for the conversion of PTK to (S)-PTE in the presence of 2-propanol as a hydrogen donor. The enzyme corresponding to the reaction was purified homogeneity, characterized and designated *Leifsonia* alcohol dehydrogenase (LSADH). The purified enzyme had a molecular weight of 110,000 and was composed of four identical subunits (molecular weight, 26,000). LSADH required NADH as a cofactor, showed little activity with NADPH, and reduced a wide variety of aldehydes and ketones. LSADH catalyzed the enantioselective reduction of some ketones with high enantiomeric excesses (e.e.): PTK to (S)-PTE (>99% e.e.), acetophenone to (R)-1-phenylethanol (99% e.e.), and 2-heptanone to (R)-2-heptanol (>99% e.e.) in the presence of 2-propanol without an additional NADH regeneration system. Therefore, it would be a useful biocatalyst.

Enantioselective organic synthesis is useful for producing chiral synthons for pharmaceuticals, agricultural chemicals, and liquid crystals. Routes to obtaining optically pure compounds include enantiomer separation from a racemic mixture, derivation of natural substances, and asymmetric synthesis. Chiral metal complexes such as BINAP-Ru have been successfully used as catalysts in a number of cases of enantioselective synthesis (18, 19). However, in many reactions, difficulties remain in attaining sufficient optical purity and productivity. To overcome the disadvantages of conventional processes, biocatalytic transformation systems using enzymes have been applied to the asymmetric synthesis of optically pure substances. Asymmetric reduction is one of the most promising processes because there is no loss of substrate, unlike racemic separation using hydrolases.

Oxidoreductases have been used in the preparation of chiral alcohols (17) with NAD⁺-dependent alcohol dehydrogenases (ADHs) from yeast, horse liver (6), *Candida parapsilosis* (20), and *Pseudomonas* sp. (2) and with NADP⁺-dependent ADHs from *Thermoanaerobium brochii* (27) and *Lactobacillus kefir* (3, 7), aldehyde reductase from *Sporobolomyces salmonicolor* (EC 1.1.1.2) (14), and carbonyl reductase (EC 1.1.1.184) from *Candida magnoliae* (24). However, they have the disadvantages of a narrow substrate specificity, insufficient stereospecificity or sensitivity to organic solvents. In addition, to overcome the bioreduction, it is necessary to regenerate NAD(P)H. Although there have been many efforts to reproduce NADH with coupling systems using formate/formate dehydrogenase (FDH), the enzyme's high cost and low activity (8) has precluded general usage. Recently, Shimizu et al. (22) and

Kataoka et al. (15) reported a recombinant enzyme system consisting of aldehyde reductase of *S. salmonicolor* or carbonyl reductase of *C. magnoliae*, coupled with an NADPH regenerating system comprising glucose/glucose dehydrogenase (GDH), and succeeded in the accumulation of (R)- and (S)-4-chloro-3-hydroxybutanoates ethyl esters from the corresponding ketone. However, GDH should be coexpressed in *Escherichia coli* to regenerate NADPH in such cases.

From the view point of NAD(P)H regeneration, 2-propanol is another suitable hydrogen donor because of its chemical properties and low cost (2, 23). Recently, Itoh et al. reported that phenylacetaldehyde reductase (PAR) from styrene-assimilating *Corynebacterium* sp. strain ST-10 (9–12, 25, 26) is a unique NADH-dependent ADH that shows a broad substrate range and a high enantioselectivity to give (S)-alcohols from various carbonyl compounds. PAR can be used for the production of various chiral alcohols, including (S)-1-phenylethanol and ethyl (R)-4-chloro-3-hydroxybutanoate and function 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 system is regarded as a superior asymmetric hydrogen-transfer reduction process. However, PAR can scarcely transform phenyl trifluoromethyl ketone (PTK) to (R)- or (S)-1-phenyltrifluoroethanol (PTE), which would be a potential chiral synthon for liquid crystals. Therefore, we performed the screening of a novel enzyme reducing PTK to (S)-PTE by using 2-propanol as a hydrogen donor, which would have the opposite stereoselectivity to PAR. A screening strategy was used to investigate microorganisms grown on gaseous styrene because, under such selective conditions, the microorganisms isolated from soil would be restricted to certain coryneform bacteria and *Pseudomonas* (9), in which useful ADHs have already been reported (2, 8).

We describe here the screening of microorganisms that can

* Corresponding author. Mailing address: Biotechnology Research Center, Toyama Prefectural University, Kosugi, Toyama 939-0398, Japan. Phone: 81-766-56-7500, ext. 560. Fax: 81-766-56-2498. E-mail: itoh@pu-toyama.ac.jp.

reduce PTK to (S)-PTE, the purification of the corresponding ADH (LSADH) from *Leifsonia* sp. strain S749, and the characterization and evaluation as an asymmetric hydrogen-transfer biocatalyst of LSADH.

MATERIALS AND METHODS

Medium and isolation procedures for styrene-degrading or styrene-resistant microorganisms. The medium for the agar plates (deep dish type, 90 by 19 mm) for screening consisted of 0.3% (wt/vol) $(\text{NH}_4)_2\text{SO}_4$, 0.3% KH_2PO_4 , 0.1% NaCl, 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.5% agar in tap water (pH 7.0) (medium A). Soil samples collected from rich fields in Toyama, Japan, were mixed with 10 ml of sterile water, and then 0.1 ml of the mixture was spread out onto an agar plate. Styrene vapor was supplied by a small open test tube (diameter by length, 6 by 30 mm) in the plate as a sole carbon source, and the plates were placed in a plastic case (9). Although the styrene gas concentration was not measured, styrene was considered to be saturated in the air at 30°C. The styrene-degrading or styrene-resistant microorganisms that could form colonies on the agar plate were cultivated under these conditions at 30°C for 3 days. The bacteria obtained by the monoclonal isolation were maintained at 4°C on LB medium (pH 7.0).

Screening of PTK-reducing strains. To facilitate handling, each strain isolated as described above was cultured in a liquid medium consisting of 0.5% yeast extract and 0.5% peptone (pH 7.0) in a test tube that was constantly shaken for 1 day at 30°C. The liquid culture broth (0.1 ml) was spread onto a plate (medium A with 0.1% yeast extract) and cultured for 3 days at 30°C under a styrene atmosphere. The cells grown on the plate were suspended in 2 ml of 20 mM potassium phosphate buffer (KPB; pH 7.0) and collected by centrifugation ($20,000 \times g$, 1 min), and then the washed cells of each microorganism were suspended in 1 ml of reaction mixture containing 50 μmol of KPB (pH 7.0), 0.5 μmol of NAD^+ , 0.5 μmol of NADP^+ , 3% (vol/vol) (392 μmol) 2-propanol, and 20 μmol of PTK. The reaction mixture in a 2-ml polypropylene tube was incubated at 30°C for 18 h with shaking in a Mini-incubator M-36 (Taitec Corp., Saitama, Japan) with shaking (2,500 rpm). After the reaction, the mixture was vigorously shaken with 1 ml of ethyl acetate for extraction. The ethyl acetate layer after drying with anhydrous Na_2SO_4 was analyzed to determine PTK and PTE contents by gas chromatography (GC) as described below.

Analysis of PTK, (S)-PTE, and (R)-PTE. Quantitative analysis of the PTK, (S)-PTE, and (R)-PTE contents was performed with a GC apparatus (HP 6890 GC system; Hewlett-Packard) equipped with a CP-cyclodextrin- β -236-N19 chiral column (0.25 mm by 25 m, 0.25- μm film; Chrompack, Midderburg, The Netherlands) and a flame ionization detector. Helium gas was used as a carrier at 15 lb/in² (0.5 ml/min), the split ratio was 50, and the injection and detection temperatures were 240 and 250°C, respectively. The column temperature was maintained isothermally at 140°C. Under these conditions, PTK, (S)-PTE, and (R)-PTE were detected at 2.3, 6.7, and 7.0 min, respectively.

Large-scale cultivation of *Leifsonia* sp. strain S749. Cultivation of *Leifsonia* sp. strain S749 was performed in a 5-liter jar fermentor containing 3 liters of medium B comprising 0.3% (wt/vol) $(\text{NH}_4)_2\text{SO}_4$, 0.3% KH_2PO_4 , 0.1% NaCl, 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2% (vol/vol) DL-1-phenylethanol, 0.25 (wt/vol) % Bacto peptone, 0.5 (wt/vol) % Bacto yeast extract, and 0.05% (vol/vol) antifoam PE-H (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (pH 7.0), with aeration at 1.5 liters/min and an agitation speed of 400 rpm for 48 h at 30°C.

Enzyme assay. LSADH activity was assayed spectrophotometrically at 25°C by measuring the decrease in the absorbance of NADH at 340 nm. The reaction mixture consisted of 3.0 μmol of PTK, 0.4 μmol of NADH, 75 μmol of KPB (pH 7.0), and 10 μl of enzyme solution in a total volume of 1.5 ml. The oxidative reaction of LSADH was also measured at 340 nm in 1.5 ml of reaction mixture containing 15 μmol of 2-propanol as a substrate, 4.5 μmol of NAD^+ , 150 μmol of KPB (pH 7.0), and 10 μl of enzyme solution. The blank contained buffer instead of substrate. One unit of enzyme was defined as the amount that converted 1 μmol of NADH in 1 min under these conditions.

Enzyme purification. All purification procedures were performed at 0 to 4°C in 20 mM KPB (pH 7.0), unless indicated otherwise. The washed cells (11 g [wet weight]) isolated from 3 liters of culture broth were suspended in 100 ml of the buffer and then disrupted with an ultrasonic oscillator (INSONATOR 201 M; Kubota Corp., Tokyo, Japan) for 30 min. After centrifugation ($13,000 \times g$, 30 min), the resulting supernatant was fractionated with solid ammonium sulfate. The precipitate obtained with 25 to 60% saturation of ammonium sulfate was collected, dialyzed against the buffer, and applied to a DEAE-Toyopearl 650 M (Tosoh Co., Ltd., Tokyo, Japan) column (2.5 by 13 cm) equilibrated with the buffer. The enzyme was eluted with a linear 0 to 1.0 M NaCl gradient in the same buffer. The fractions with high enzyme activity were collected (total volume of 20

ml). The solution mixed with ammonium sulfate up to a concentration of 1.0 M was applied to a Butyl-Toyopearl 650 M (Tosoh) column (2.5 by 13 cm) which had been equilibrated with 1.0 M ammonium sulfate in 20 mM buffer (pH 7.0). The enzyme was eluted with a linear 1.0 to 0 M ammonium sulfate gradient in the buffer. The collected fractions (total volume of 28 ml) with high enzyme activity were concentrated to 1 ml by a Centriprep YM-30 (cutoff molecular weight of 30,000; Millipore). The enzyme solution was applied to a Cellulofine GCL-2000-sf (Seikagaku Corp., Tokyo, Japan) gel filtration column (2 by 100 cm) equilibrated with the buffer, and the elution was performed at a flow rate of 0.3 ml/min. The fractions with high enzyme activity were collected (total volume of 8 ml), and then the enzyme was loaded onto a Bioassit Q (Tosoh) column (4.6 mm by 5 cm) which had been equilibrated with 20 mM Tris-HCl (pH 8.0) and was connected to an analytical high-performance liquid chromatography (HPLC) system. The enzyme was eluted with a linear 0 to 0.8 M NaCl gradient in the same buffer at a flow rate of 0.2 ml/min. The fractions (2 ml) with high enzyme activity were collected and desalted by using the Centriprep YM-30. The enzyme solution thus obtained was used as the purified enzyme for characterization.

Protein assay. The protein concentration was estimated by measuring the absorbance at 280 nm or by using the method of Bradford, calibrated with bovine serum albumin as a standard (Bio-Rad Protein Assay Kit; Bio-Rad).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12.7% polyacrylamide slab gel with the Tris-glycine buffer system described by Laemmli (16). The molecular mass of the enzyme subunit was determined from the relative mobility of standard proteins.

Partial NH_2 -terminal amino acid sequences of LSADH. The enzyme was electrophoresed on an SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane (Bio-Rad) by using a semidry electroblotting apparatus (NA-1512; Nippon Eido, Tokyo, Japan) at a constant current of 0.8 mA/cm² gel for 90 min by the method of Hirano and Watanabe (5), and then stained with Coomassie brilliant blue G-250. The amino acid sequence at the N terminal end of the enzyme on the polyvinylidene difluoride membrane was determined by using a HP G1005A protein sequencing system (Hewlett-Packard).

Molecular weight. The molecular weight of the enzyme was determined by analytical HPLC with a TSK-Gel G3000SW_{XL} (Tosoh) column (7.8 mm by 30 cm) at a flow rate of 0.8 ml/min with 50 mM Tris-HCl (pH 7.0) containing 0.1 M NaCl. The molecular mass of the native enzyme was determined by comparing the retention time with those of standard proteins.

Determination of substrate specificity of LSADH. The substrate specificity of LSADH was determined spectrophotometrically by measuring the decrease in absorbance of NADH at 340 nm. The reaction conditions were the same as those for the LSADH assay system, except that different substrate and enzyme concentrations were used.

Kinetic analysis. A steady-state kinetic analysis of the LSADH reaction was performed in 100 mM KPB (pH 7.0). To determine the apparent K_m value for PTK, its concentration was varied from 2.0 to 20 mM in the presence of a fixed concentration of NADH (0.27 mM). In the same way, to determine the apparent K_m value for NADH, its concentration was varied from 2.1×10^{-3} to 0.27 mM in the presence of 10 mM PTK. The apparent K_m value for 2-propanol in the oxidative reaction was measured by varying its concentration from 50 to 250 mM in the presence of 3 mM NAD^+ , and the apparent K_m value for NAD^+ was determined by varying its concentration from 5.8×10^{-3} to 3.0 mM in the presence of a fixed concentration of 2-propanol (10 mM).

Enantioselective reduction of PTK, acetophenone, and 2-heptanone. The reaction mixture consisted of 0.1 mmol KPB (pH 7.0), 10 mg of each substrate, 1 μmol of NAD^+ , 5% (vol/vol) (653 μmol) 2-propanol, and 1 U of purified LSADH in a total volume of 1 ml. The reaction proceeded 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 anhydrous Na_2SO_4 and used for the analysis. The conversion yield and enantiomeric purity of the product were determined on the basis of the peak areas of ketone substrates and alcohol products on GC as described above. Acetophenone, (R)-1-phenylethanol, and (S)-1-phenylethanol were analyzed by GC in the same manner as PTK, (S)-PTE, and (R)-PTE except that the column temperature was 120°C. Acetophenone, (R)-1-phenylethanol, and (S)-1-phenylethanol were detected at 4.3, 6.3, and 6.6 min, respectively.

2-Heptanone and 2-heptanol were analyzed by GC using a Shimadzu GC-18A system equipped with a capillary column (DB-1, 0.25 mm by 30 m; J & W Scientific, CA) with a flame ionization detector. GC was carried out under the following conditions: a column temperature of 60°C, injection and detection temperatures of 250°C, and a flow rate of 1 ml min⁻¹ of He. The retention time was 6.5 min for 2-heptanone and 7.3 min for 2-heptanol. The product was extracted with ethyl acetate from the reaction mixture, dried with anhydrous

FIG. 1. Screening for PTK-reducing microorganisms with the resting-cell reaction. Strain S749 (●) was selected as the best producer of (S)-PTE under the conditions tested.

Na_2SO_4 and evaporated, subjected to silica gel chromatography (MERCK), and eluted with *n*-hexane–2-propanol (49:1). The product was obtained after the evaporation of fractions containing the product to give clear oil. Since the chemical modification was necessary to determine the absolute configuration of 2-heptanol, the purified product was converted into a benzoyl derivative by benzoyl chloride as described in a previous paper (11). The absolute configuration of the benzoyl derivative of 2-heptanol was determined by HPLC on a Chiralcel OB-H column (Daicel Chemical Industries, Ltd., Osaka, Japan) under the following conditions: hexane–2-propanol (49:1) (mobile phase), flow rate of 0.5 ml/min (30°C), detection at 254 nm, and retention time of 7.2 min for the (R)-derivative and 7.9 min for the (S)-derivative.

Chemicals. SDS-PAGE molecular weight standards (low range) were purchased from Nippon Bio-Rad Laboratories, Tokyo, Japan. The marker protein kit for HPLC was obtained from Oriental Yeast Co., Ltd., Tokyo, Japan. PTK was purchased from Tokyo Kasei Co., Ltd., Tokyo, Japan, and (S)-1-PTE was from Aldrich Chemical Co. 2,3'-Dichloroacetophenone, 3',4'-dimethoxyacetophenone, methyl 4-bromo-3-oxobutanoate, and ethyl 4-bromo-3-oxobutanoate were generous gifts from Sumitomo Chemical Co., Ltd., Osaka, Japan. All other reagents were of analytical grade.

RESULTS

Screening of PTK-reducing microorganisms. In the preliminary experiments with some styrene-assimilating microorganisms, we confirmed that the resting-cell reaction with 3% (vol/vol) 2-propanol functioned well in detecting PTK-reducing enzyme. As shown in Fig. 1, PTK-reducing activity was found to be widely distributed in styrene-assimilating or styrene-resistant microorganisms that mainly belong to gram-positive bacterial genera such as *Corynebacterium*, *Rhodococcus*, and the gram-negative genus *Pseudomonas* (9). Moreover, the data

indicated that these bacteria had a tendency to convert PTK to (S)-PTE. We observed that ca. 60% of the microorganisms isolated (900 strains) could reduce PTK to PTE with a more than 10% conversion. One strain (strain S749) (see Fig. 1) was selected as the best producer of (S)-PTE under the conditions tested; the optical purity of the (S)-PTE produced by the resting reaction with strain S749 was 87% enantiomeric excesses (e.e.), and the molar yield was 86%.

Identification of the isolated microorganism. Strain S749 was a coryneform gram-positive bacterium (0.6 by ca. 1.0 to 2.0 μm) and showed the following physiological features: yellow colonies on nutrient agar, growth at 37°C (+ weak) but not at 45°C, motility, negative for oxidase, positive for catalase, no spore formation, and positive or negative results for the O-F test. In addition, an analysis of 16S rRNA performed by NCIMB Japan (Shizuoka, Japan) showed that the microorganism was closely related to *Leifsonia aquatica* DSM20146 (German Collection of Microorganisms and Cell Cultures) (formerly *Corynebacterium aquatica*) (99.6%), *L. aquatica* JCM1368 (Japan Collection of Microorganisms) (99.2%), and *L. xyl* JCM9733 (98.5%). From these results, including the physiological properties, the microorganism was identified as *Leifsonia* sp. strain S749.

Purification of LSADH. Purification procedures for LSADH are summarized in Table 1. LSADH was purified to homogeneity 49-fold from the cell extract by sequential column chromatographies. Preliminary experiments showed that the enzyme did not bind to dye-ligand matrices such as Blue-Sepharose and Red-Sepharose, although it is an NADH-dependent oxidoreductase. Purified LSADH (0.33 mg) showing 10.3 U/mg of protein was obtained from 11 g (wet weight) of cells (3-liter culture broth) with a yield of 3.2%. The purity of the enzyme was checked by SDS-PAGE (Fig. 2) and analytical HPLC with TSK Gel 3000SW_{XL}. The enzyme sample was considered homogeneous on the basis of these analyses.

Molecular weight and subunit structure. According to the analytical HPLC on TSK Gel 3000SW_{XL}, the molecular mass of the enzyme was estimated to be 110,000 Da. SDS-PAGE revealed a single band, and the subunit molecular mass was 26,000 Da. The data showed that LSADH was a tetramer protein comprising subunits of identical molecular weight.

Partial N-terminal amino acid sequence. The N-terminal amino acid sequence of the enzyme was determined to be Ala-Gln-Tyr-Asp-Val-Ala-Asp-Arg-Ser-Ala-Ile-Val-Thr-Gly-Gly.

Spectral properties. The absorption spectrum of the enzyme had a maximum at 279 nm. No absorbance was detected at wavelengths longer than 320 nm. Thus, the enzyme contained no flavin or pyrroloquinoline quinone, which are commonly

TABLE 1. Purification of LSADH from *Leifsonia* sp. strain S749

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification factor (fold)
Crude extract	499	105	0.21	100	1
Ammonium sulfate precipitation	272	40.5	0.15	38.6	0.7
DEAE-Toyopearl	94.6	33.8	0.36	32.2	1.7
Butyl-Toyopearl	15.3	16.7	1.09	15.9	5.2
Cellulofine GCL-2000sf	5.20	11.6	2.23	11.0	10.6
Bioassist Q	0.33	3.4	10.3	3.2	49.0

FIG. 2. SDS-PAGE of the LSADH from *Leifsonia* sp. strain S749. Lane A, molecular weight standards, including (from top to bottom) phosphorylase B (M_r , 97,400), serum albumin (M_r , 66,200), ovalbumin (M_r , 45,000), carbonic anhydrase (M_r , 31,000), trypsin inhibitor (M_r , 21,500), and lysozyme (M_r , 14,400); lane B, purified enzyme ($\sim 6 \mu\text{g}$). The gel was stained by Quick-CBB (Wako Pure Chemicals, Osaka, Japan).

present in NAD(P)H (quinone acceptor) dehydrogenase (4) (EC 1.6.99.2) and in quinoprotein dehydrogenases (1).

Substrate specificity of LSADH. LSADH used an NADH as a coenzyme, and only 5% of the activity was observed for NADPH. Therefore, LSADH was an NADH-dependent oxidoreductase. Table 2 shows the relative activity for the reductive reaction of LSADH with NADH for some aldehydes, ketones, and keto esters when the activity for PTK is 100%. LSADH catalyzed the reduction of various aldehydes, ketones, and keto esters. The enzyme did not act on short-chain alkyl aldehydes, including formaldehyde and acetaldehyde; however, it showed strong activity toward medium-chain normal alkyl aldehydes of between C_5 and C_8 . The highest activity was observed with *n*-hexyl aldehyde (1,029% compared to the activity of PTK). Although benzaldehyde and phenylacetaldehyde were not suitable substrates for the enzyme, 3-phenylpropionaldehyde served as a good substrate. LSADH did not apparently catalyze the reduction of short-chain alkyl ketones such as acetone and 2-butanone at low concentrations. On the other hand, it showed strong activity toward medium-chain normal 2-ketoalkanes. The highest level of activity was observed with 2-heptanone (229% compared to the activity of PTK). The substrate spectra observed for ketones were similar to those for aldehydes. For arylketones, acetophenone was reduced to 1-phenylethanol with a relative activity of 6% of that of PTK. 3'- and 4'-Halogenated acetophenones were more efficiently catalyzed by LSADH. Compared to 3'- and

TABLE 2. Substrate specificity of LSADH for reductive reaction measured by the NADH consumption

Substrate	Relative activity (%) ^a
Aldehydes	
Formaldehyde	0
Acetaldehyde	0
Propionaldehyde	2
<i>n</i> -Butyraldehyde	10
<i>n</i> -Valeraldehyde	132
<i>n</i> -Hexyl aldehyde	1,029
<i>n</i> -Heptyl aldehyde	755
<i>n</i> -Capryl aldehyde	435
Benzaldehyde	13
Phenylacetaldehyde	8
3-Phenylpropionaldehyde	1,218
<i>trans</i> -Cinnamaldehyde	1,078
Ketones	
Acetone	0
Chloroacetone	238
1,1-Dichloroacetone	1,078
2-Butanone	0
3-Chloro-2-butanone	151
4-Hydroxy-2-butanone	26
2-Pentanone	17
2-Hexanone	104
2-Heptanone	229
2-Octanone	147
2-Nonanone	110
3-Pentanone	3
PTK	100
Acetophenone	6
2-Chloroacetophenone	29*
2-Bromoacetophenone	3*
2-Hydroxyacetophenone	0
2'-Chloroacetophenone	0
2'-Bromoacetophenone	2
3'-Chloroacetophenone	70
3'-Bromoacetophenone	151
4'-Chloroacetophenone	60
4'-Bromoacetophenone	77
3'-Methoxyacetophenone	51
2,3'-Dichloroacetophenone	67*
3',4'-Dimethoxyphenylacetone	24
1-Phenyl-1-butanone	0
1-Phenyl-2-butanone	12
1-Phenyl-3-butanone	353
Cyclopentanone	1
Cyclohexanone	0
Keto esters	
Ethyl pyruvate	488
Ethyl 3-methyl-2-oxobutylate	33
Methyl benzoylformate	1
Ethyl benzoylformate	3
Methyl 3-oxobutanoate	130
Ethyl 3-oxobutanoate	309
<i>tert</i> -Butyl 3-oxobutanoate	568
Ethyl 4-chloro-3-oxobutanoate	809
Methyl 4-bromo-3-oxobutanoate	164
Ethyl 4-bromo-3-oxobutanoate	511

^a *, substrate was dissolved in dimethyl sulfoxide and added to the reaction mixture (2 mM, 1% [vol/vol] dimethyl sulfoxide).

4'-halogenated derivatives, quite weak or no activity was observed for 2'-halogenated acetophenones. Although LSADH hardly acted on 2(α)-substituted acetophenones, such as 2-bromoacetophenone (phenacyl bromide; 3% compared to the

TABLE 3. Substrate specificity of LSADH for oxidative reaction measured by the NADH formation

Substrate	Relative activity (%)
Alkyl alcohols	
Methanol	0
Ethanol	0
1-Propanol	0
1-Butanol	0
1-Pentanol	6
1-Hexanol	44
1-Heptanol	56
1-Octanol	33 ^a
2-Propanol	100
(<i>R</i>)-2-Butanol	275
(<i>S</i>)-2-Butanol	50
(<i>R</i>)-2-Pentanol	975
(<i>S</i>)-2-Pentanol	25
(<i>R</i>)-2-Hexanol	1,906
(<i>S</i>)-2-Hexanol	38
(<i>R</i>)-2-Heptanol	2,469
(<i>S</i>)-2-Heptanol	331
(<i>R</i>)-2-Octanol	2,406
(<i>S</i>)-2-Octanol	81
3-Heptanol	93
4-Heptanol	0
Aryl alcohols	
(<i>S</i>)-1-Phenyltrifluoroethanol	0
(<i>R,S</i>)-1-Phenyltrifluoroethanol	0
(<i>R</i>)-1-Phenylethanol	87
(<i>S</i>)-1-Phenylethanol	0
2-Phenylethanol	13
Cycloalcohols	
Cyclopentanol	13
Cyclohexanol	0

^a The 1-octanol concentration was 5 mM because of its low solubility.

activity of PTK) and 2-hydroxyacetophenone (0%), 2,3'-dichloroacetophenone was reduced. 1-Phenyl-1-butanone and 1-phenyl-2-butanone were not suitable substrates for the enzyme; however, 1-phenyl-3-butanone served as a good substrate for LSADH. The activity toward cyclopentanone was quite weak. LSADH also catalyzed the reduction of α - and β -keto esters such as ethyl pyruvate, ethyl 3-methyl-2-oxobutyrate, methyl 3-oxobutanoate, ethyl 3-oxobutanoate, *tert*-butyl 3-oxobutanoate, ethyl 4-chloro-3-oxobutanoate, and ethyl 4-bromo-3-oxobutanoate with strong activity. The results revealed that LSADH has a broad substrate spectrum.

Table 3 shows the substrate specificity for the oxidative reaction of LSADH with NAD⁺ for some alcohols, when that for 2-propanol is 100%. The enzyme did not act on short-chain normal alkyl alcohols, including methanol and ethanol; however, it showed strong activity toward medium-chain normal alkyl alcohols of between C₅ and C₈. With regard to secondary alcohols, LSADH acted on (*R*)-form alkanols with a much higher level of activity than on (*S*)-form alkanols. As in the case of 1-phenylethanol, LSADH acted only on the (*R*)-form, the (*S*)-form being inert. (*S*)- and racemic 1-phenyltrifluoroethanols (PTE) were both inert for the oxidation of LSADH. The results were consistent with the observation that LSADH could reduce various ketones into (*R*)-form alcohols as described in the section below.

TABLE 4. Steady-state kinetic constants of LSADH

Substrate	K_m (mM) ^a	V_{max} (U mg ⁻¹)	V_{max}/K_m (U mg ⁻¹ mM ⁻¹)
PTK	13.6	76.6	5.63
NADH	0.048		
2-Propanol	57.5	25.7	0.45
NAD ⁺	0.12		

^a The activity was measured at pH 7.0 in both reactions.

Kinetic properties of the LSADH reaction. The K_m values of LSADH toward PTK and NADH in the reductive reaction were calculated from the Lineweaver-Burk plot to be 13.6 and 0.048 mM, respectively. The K_m values of the enzyme toward 2-propanol and NAD⁺ in the oxidative reaction were 57.5 and 0.12 mM, respectively. In all cases, regular saturation curves of the activity versus the substrate concentration were observed. As summarized in Table 4, the V_{max} value of LSADH toward 2-propanol was high enough to catalyze the oxidation of 2-propanol to acetone and reduction of NAD⁺ to NADH when it was sufficiently supplied in the reaction mixture in spite of the enzyme's high K_m value. The catalytic efficiency of the LSADH reaction calculated as V_{max}/K_m for 2-propanol was comparable with that for PTK (Table 4).

Effect of various compounds on the LSADH reaction. The effect of various compounds on the LSADH reaction was examined by adding each compound to the reaction mixture (Table 5). Metal ions such as Pb²⁺, Ag⁺, and Hg²⁺ inhibited the LSADH reaction, as did Fe³⁺, Cu²⁺, and CN⁻ slightly. Mg²⁺ and Mn²⁺ essential for the activity of *Lactobacillus kefir* ADH (7, 8) had no effect on LSADH. In general, LSADH was resistant to various additive substances.

Effect of temperature on enzyme activity and stability. The enzyme activity was measured at various temperatures, with the optimum temperature observed ca. 50°C at pH 7.0: 23% activity at 20°C, 50% at 30°C, 90% at 40 and 60°C, 100% at 50°C, and 40% at 70°C. The thermal stability of LSADH was also examined by incubation for 1 h in the 20 mM Tris-HCl buffer (pH 7.0). The enzyme was stable at <30°C and retained 80% activity at 40°C, 70% activity at 50°C, and 24% activity at 60°C but 0% activity at above 70°C.

Effect of pH on enzyme activity and stability. The effect of pH on the activity was measured in the following buffers (final concentration, 0.1 M): citrate-K₂HPO₄ (pH 4.0 to 5.5), KPB (pH 5.5 to 7.5), Tris-HCl (pH 7.5 to 9.0), and glycine-NaOH buffer (pH 8.0 to 10.0). In the reductive reaction, the enzyme showed maximum activity at pH 6.0. On the other hand, the enzyme showed maximum activity at pH 9.5 in the oxidative reaction (Fig. 3). In both reactions, LSADH showed a high level of activity over a wide range of pH. The pH stability of LSADH was also measured after incubation in one of the buffers described above at 25°C for 1 h. The enzyme was stable in a pH range from 5.0 to 9.5.

Stereospecificity of LEADH and its evaluation as an asymmetric hydrogen-transfer biocatalyst. For determining the stereospecificity of LSADH reduction, the absolute configurations of the alcohol products were measured. Figure 4 indicates the data on PTK reduction; PTK was completely converted into (*S*)-PTE after 24 h with a molar yield of 100% and an

TABLE 5. Effect of various compounds on LSADH

Compound	Concn (mM)	Relative activity (%)
None		100
Metal ions		
LiCl	1	100
NaCl	1	98
MgCl ₂	1	98
CaCl ₂	1	110
MnCl ₂	1	100
FeCl ₃	1	66
CoCl ₂	1	110
NiCl ₂	1	105
ZnCl ₂	1	90
CuCl ₂	1	83
PbCl ₂	1	37
AgNO ₃	0.1	34
BaCl ₂	1	100
HgCl ₂	0.1	15
Chelators		
EDTA	1	93
TTHA ^a	1	88
2,2'-Bipyridyl	1	102
Sulfhydryl reagents		
<i>p</i> -Chloromercuribenzoic acid	1	91
<i>N</i> -Ethylmaleimide	1	102
Iodoacetate	1	100
Reducing agents		
2-Mercaptoethanol	1	98
Dithiothreitol	1	102
Others		
Hydrazine	0.1	95
Phenylmethylsulfonyl fluoride	1	82
Hydroxylammonium chloride	1	98
NaN ₃	1	100
KCN	1	59
Pyrazol	10	90

^a TTHA, triethylenetetramine-*N,N,N',N'',N''',N''''*-hexaacetic acid.

optical purity of >99% e.e. As exemplified in Table 6, the enantioselectivities of LSADH were high enough to produce optically pure alcohols. In the case of acetophenone, it was converted to (*R*)-1-phenylethanol with a molar yield of 79% and an optical purity of 99% e.e. 2-Heptanone was also reduced to (*R*)-2-heptanol with a molar yield of 78% and an optical purity of >99% e.e. The stereoselectivity of the LSADH reaction well coincided with the substrate specificity of LSADH for the oxidative reaction for 1-phenylethanol and 2-heptanol: its specific activity for the (*R*)-form (Table 3). In addition, good molar yields observed for three substrates indicated that efficient NADH-regeneration occurred during the reaction without the additional cofactor regenerating system, as illustrated in Fig. 5.

DISCUSSION

In this study, we describe a screening for microorganisms that could produce (*S*)-PTE from PTK among styrene-assimilating microorganisms in the presence of 2-propanol as a hydrogen donor. Although strain S749 was not able to assimilate

FIG. 3. LSADH activity as a function of pH in the reduction of PTK and oxidation of 2-propanol. The activity was measured in the following 0.1 M buffers: citrate-K₂HPO₄ (pH 4.0 to 5.5) (○), KPB (pH 5.5 to 7.5) (△), Tris-HCl (pH 7.5 to 9.0) (□) in the reductive reaction, citrate-K₂HPO₄ (pH 4.0–5.5) (●), KPB (pH 5.5 to 7.5) (▲), Tris-HCl (pH 7.5 to 9.0) (■), and glycine-NaOH (pH 9.0 to 11.0) (◆) in the oxidative reaction. The remaining activity of LSADH was also measured after incubation in one of the buffers at 25°C for 1 h (dashed line).

styrene as a sole source of carbon and energy, it could form colonies on agar plates saturated with styrene gas. This screening method could preferentially isolate bacteria belonging to *Corynebacterium*, *Rhodococcus*, *Pseudomonas*, and related

FIG. 4. GC of PTE enantiomers. (A) Racemic PTE and PTK standard; (B) enzymatically produced (*S*)-PTE. The sample was analyzed under the conditions described in Materials and Methods.

TABLE 6. Enantioselective reduction of PTK, acetophenone, and 2-heptanone by LSADH

Substrate	Relative activity (%)	Structure of product	Type, e.e. (%)	Molar conversion yield (%) ^a
PTK	100		(<i>S</i>)-form, >99	100
Acetophenone	6		(<i>R</i>)-form, 99	79
2-Heptanone	229		(<i>R</i>)-form, >99	78

^a Not optimized (reaction time of 24 h).

strains (9). The S749 strain was identified as *Leifsonia* sp. and very similar to *L. aquatica* (formerly *Corynebacterium aquatica*). In previous studies (10–12), Itoh et al. reported PAR in the styrene-assimilating *Corynebacterium* sp. strain ST-10 and established a practical hydrogen-transfer bioreduction process to produce (*S*)-1-phenylethanol and other chiral alcohols from the corresponding ketones. On screening with the resting-cell reaction, we adopted a reaction mixture containing 3% (vol/vol) 2-propanol in order to find an enzyme which could regenerate NADH by transferring hydrogen from 2-propanol without an additional coenzyme regenerating system such as formate/FDH and glucose/GDH. Under the conditions adopted, 2-propanol must play a role in increasing permeability of the cell membrane for substrate transfer, which made the screening operation quite easy. We succeeded in finding ADH in *Leifsonia* sp., which could reduce PTK into (*S*)-PTE using 2-propanol as a hydrogen donor. Due to the high ratio of microorganisms converting PTK (Fig. 1), our screening strategy efficiently detected a PTK-reducing biocatalyst.

LSADH productivity in *Leifsonia* sp. strain S749 was six times higher when DL-1-phenylethanol was added to the liquid culture medium (data not shown). It was also observed that DL-1-phenylethanol was not used as a sole carbon source but was co-oxidized when other nutrients were added to the medium. Therefore, LSADH might physiologically participate in the conversion of aromatic carbonyl compounds.

LSADH was purified to homogeneity on SDS-PAGE from the cell extract of *Leifsonia* sp. strain S749 by sequential column chromatographies. The purified enzyme consisted of four subunits (all 26,000 Da). The N-terminal amino acid sequence of LSADH (Ala-Gln-Tyr-Asp-Val-Ala-Asp-Arg-Ser-Ala-Ile-Val-Thr-Gly-Gly) showed similarity to that of *Mesorhizobium loti* 3-oxoacyl-acyl carrier protein reductase protein (2705259LRD; 69% identity), *Streptomyces* polyketide reductase protein (L34880-3; 75%) and *Bradyrhizobium japonicum* short-chain dehydrogenase protein (AP005941-253; 61%) (data not shown), which consist of 239, 262, and 256 amino acid residues, respectively. Thus, the result suggested that LSADH probably belongs to the short-chain dehydrogenase/

FIG. 5. Asymmetric hydrogen-transfer bioreduction by LSADH with 2-propanol as a hydrogen donor.

TABLE 7. Comparison of biochemical properties of ADHs producing (R)-form alcohol

Property	LSADH	<i>L. kefir</i> ADH ^a	<i>Pseudomonas</i> ADH ^b
Classification	Probable short-chain ADH	Short-chain ADH	Short-chain ADH
Molecular mass	110 kDa, homotetramer	105 kDa, homotetramer	ND ^c
Metal requirement	Probably none	Mg ²⁺ (Mn ²⁺)	ND
Coenzyme	NAD ⁺	NADP ⁺	NAD ⁺
K_m			
For coenzyme	0.12 mM (NAD ⁺), 48 μ M (NADH)	0.19 mM (NADP ⁺), 0.13mM (NADPH)	0.525 mM (NAD ⁺)
For 2-propanol	57.5 mM	0.12 mM	75 μ M
Stereospecificity			
(R)-1-Phenylethanol	99% e.e.	ND	94% e.e.
(S)-PTE	>99% e.e.	>99% e.e.	92% e.e.

^a Data from references 3, 7, and 8.^b Data from references 2 and 8.^c ND, not determined.

reductase family. The short-chain dehydrogenase/reductase family comprises oxidoreductases that catalyze oxidation/reduction with NAD(P)H as a cofactor, active as a dimer- or tetramer-protein, and each monomer consists of approximately 250 amino acid residues (13). LSADH consisted of four subunits of the same size and existed as a tetramer protein, supporting that LSADH from *Leifsonia* sp. strain S749 belongs to this family.

As shown in Table 2, LSADH catalyzed the reduction of various ketones, especially 2-ketoalkanes, halogenated acetophenones, and α - and β -keto esters, to give the corresponding alcohols. The stereoisomers of these alcohols are important starting materials for the synthesis of pharmaceuticals, agrochemicals, and liquid crystals. LSADH converted PTK, acetophenone, and 2-heptanone into (S)-PTE, (R)-1-phenylethanol, and (R)-2-heptanol, respectively, with high optical purities (Table 6). Therefore, the hydride anion of NADH is probably transferred to the *si* face of the carbonyl of acetophenone, suggesting that the enantioselectivity of LSADH contradicts against to Prelog's rule (21).

Comparison data of (R)-form alcohol-producing ADHs are summarized in Table 7. Although the available data were limited (2, 3), the substrate specificity of LSADH appeared to be comparatively similar to that of *L. kefir* ADH and differed from that of *Pseudomonas* ADH. In terms of coenzyme dependency and the Mg²⁺ requirement, however, LSADH differed from *L. kefir* ADH. Imperfect data concerning the *lsadh* gene (data not shown) suggested that LSADH probably does not contain any metals such as zinc. K_m values for NAD⁺ and 2-propanol of LSADH apparently differed from those of *Pseudomonas* ADH, and stereoselectivities toward acetophenone and PTK were superior to those of *Pseudomonas* ADH. The results indicated that LSADH is a unique ADH compared to those previously reported.

Thus, LSADH would be a useful biocatalyst for obtaining some optically pure alkyl and aryl alcohols, considering its stereoselectivity, (R)-1-phenylethanol from acetophenone, broad substrate spectrum, NADH dependency, and stability over a wide pH range. Moreover, as shown in Tables 3 and 4, LSADH could catalyze the oxidation of some alcohols, includ-

ing 2-propanol, 2-butanol, and cyclopentanol. We demonstrated that LSADH could function as a biocatalyst to transfer hydrogen from a donor (2-propanol) to various ketones through a smooth NADH-regeneration (Tables 4 and 6). It has also been determined that LSADH is stable and has a practical level of activity in a 2-propanol concentration ranging from 0 to 15 (vol/vol) % (data not shown). We expect the bioreduction process using recombinant LSADH to be a practical way to produce a large amount of optically pure alcohol.

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