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Characterization of an Allylic/Benzyl Alcohol Dehydrogenase from *Yokenella* sp. Strain WZY002, an Organism Potentially Useful for the Synthesis of α,β-Unsaturated Alcohols from Allylic Aldehydes and Ketones

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A novel whole-cell biocatalyst with high allylic alcohol-oxidizing activities was screened and identified as *Yokenella* sp. WZY002, which chemoselectively reduced the C═O bond of allylic aldehydes/ketones to the corresponding α,β-unsaturated alcohols at 30°C and pH 8.0. The strain also had the capacity of stereoselectively reducing aromatic ketones to (*S*)-enantioselective alcohols. The enzyme responsible for the predominant allylic/benzyl alcohol dehydrogenase activity was purified to homogeneity and designated YsADH (alcohol dehydrogenase from *Yokenella* sp.), which had a calculated subunit molecular mass of 36,411 Da. The gene encoding YsADH was subsequently expressed in *Escherichia coli*, and the purified recombinant YsADH protein was characterized. The enzyme strictly required NADP(H) as a coenzyme and was putatively zinc dependent. The optimal pH and temperature for crotonaldehyde reduction were pH 6.5 and 65°C, whereas those for crotyl alcohol oxidation were pH 8.0 and 55°C. The enzyme showed moderate thermostability, with a half-life of 6.2 h at 55°C. It was robust in the presence of organic solvents and retained 87.5% of the initial activity after 24 h of incubation with 20% (vol/vol) dimethyl sulfoxide. The enzyme preferentially catalyzed allylic/benzyl aldehydes as the substrate in the reduction of aldehydes/ketones and yielded the highest activity of 427 U mg^{-1} for benzaldehyde reduction, while the alcohol oxidation reaction demonstrated the maximum activity of 79.9 U mg^{-1} using crotyl alcohol as the substrate. Moreover, kinetic parameters of the enzyme showed lower K_m values and higher catalytic efficiency for crotonaldehyde/benzaldehyde and NADPH than for crotyl alcohol/benzyl alcohol and NADP$^+$, suggesting the nature of being an aldehyde reductase.

In the manufacture of fine chemicals, pharmaceuticals, and fragrances, α,β-unsaturated alcohols are used through selective hydrogenation of α,β-unsaturated aldehydes or ketones with the aide of chemical catalysts (1). The thermodynamics of the reduction of α,β-unsaturated aldehydes and ketones favors hydrogenation of the C═C rather than C═O bond to form saturated compounds. Consequently, hydrogenation of α,β-unsaturated aldehydes or ketones to form the corresponding unsaturated alcohols is difficult (2–4). As an alternative to a purely chemical approach, biocatalysis is increasingly preferred to achieve highly selective reductions under mild conditions (5, 6) and, when required, the reverse reactions.

Whole-cell biocatalysis for the reduction of saturated carbonyl compounds has been extensively studied due to advantages such as enzyme stability, coenzyme recycling, and the efficiency of multistep bioconversion (7). Even so, the whole-cell catalyzed reduction of α,β-unsaturated carbonyl compounds can be complex and hampered by competing enzymatic reactions (8). Thus, in the asymmetric bioreduction of citral to the α,β-saturated aldehyde citronellal, the undesirable by-products nerol, geraniol, and citronellol were formed due to the action of competing alcohol dehydrogenases and citral lyase activity (9); in stereoselective biocatalytic reduction of α-ionone by *Glomerella cingulata*, (6*S*,9*R*)-α-ionol was produced, some of which was subsequently hydrogenated by enoate reductase to form (6*S*,9*R*)-7,8-dyhidro-α-ionol (10); and with use of α,β-unsaturated aldehydes as the substrate, the aldehyde oxidation catalyzed by aldehyde dehydrogenase usually led to the formation of undesirable α,β-unsaturated carboxylic acids (11, 12). Therefore, novel biocatalysts with high chemoselectivity are needed for synthesis of α,β-unsaturated alcohols.

The key enzymes responsible for the reduction of α,β-unsaturated aldehydes and ketones to the corresponding unsaturated alcohols are allylic alcohol dehydrogenases. These enzymes from various microorganisms, such as *Acinetobacter calcoaceticus* (13), *Acinetobacter baylyi* (14), *Pseudomonas putida* (15), and *Saccharomyces cerevisiae* (16), have been characterized. All belong to the family of zinc-containing medium-chain alcohol dehydrogenase and are NAD(P) dependent. Besides allylic aldehydes and ketones, this family of enzymes also preferentially catalyzes the interconversion between benzyl aldehydes and their corresponding alcohols (17). These characterized enzymes are maximally active at ambient temperatures and are inhibited by their own substrates. This limits their usefulness because industrial biocatalysts must tolerate harsh environmental conditions, such as high temperatures and high concentrations of substrates (18). Thus, more-

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robust enzymes are needed to meet the requirements of biotechnological applications.

In this study, a biocatalytic organism, *Yokenella* sp. strain WZY002, was isolated from soil samples, and its chemoselectivity and activity in the reduction of α,β-unsaturated aldehydes or ketones to unsaturated alcohols were determined. Subsequently, alcohol dehydrogenase from *Yokenella* sp. WZY002 (YsADH) was purified, and its encoding gene was functionally expressed in *Escherichia coli* BL21(DE3). Both *Yokenella* sp. WZY002 and YsADH from recombinant *E. coli* were characterized to allow assessment of their potential use for the biosynthesis of α,β-unsaturated alcohols from allylic aldehydes and ketones.

MATERIALS AND METHODS

Materials. Bacteria with allylic alcohol dehydrogenase activities were isolated from soil from Hubei province, China. Chemicals of analytical grade were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) or Shanghai Jingchun Reagent Co., Ltd. (Shanghai, China). Restriction enzymes and TransStrat *Taq* DNA polymerase were purchased from TaKaRa Bio Inc. (Dalian, China) and TransGen Biotech Co., Ltd. (Beijing, China), respectively.

Organism, plasmid, and media. The organism used in this study, *Yokenella* sp. WZY002, has been deposited in the China Center for Type Culture Collection (CCTCC M 2013099, Wuhan, China). Cultures of the organism were grown routinely in Luria-Bertani (LB) medium at 30°C for 24 h. The pEASY-E1 expression vector from TransGen Biotech Co., Ltd. (Beijing, China), was used for overexpression of the YsADH-encoding gene, and the *E. coli* strain BL21(DE3) was used as the host. Recombinant *E. coli* was cultivated at 37°C or 20°C in LB medium containing 100 μg ml^{-1} of ampicillin.

Screening of isolates. Screening of isolates from soil was carried out according to previously reported procedures (19) with some modifications. Each isolate was purified by streaking on a plate of LB agar spread with 150 μl crotyl alcohol that was incubated at 30°C for 48 h. Isolated colonies picked from the plates were each resuspended in 60 μl sterile water. The resulting cell suspensions were subjected to NAD(P)H fluorescence high-throughput screening in a 96-well Fluotrac 200 black plate (Greiner, Germany), each well of which was filled with 300 μl of a reaction solution containing 50 mM glycine-NaOH buffer (pH 10.0), 0.5% (wt/vol) crotyl alcohol, 1 mM $NADP^+$, and 25 μl of a suspension. A well without crotyl alcohol served as the control. After incubation at 30°C for 16 h, the microplate was examined for NADPH by fluorescence spectroscopy at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Cultures that gave a fluorescence intensity increase of >400 were regarded as indicative of high allyl alcohol dehydrogenase activity of the isolate. These isolates were further tested for their oxidation of crotyl alcohol in reaction mixtures that each contained 2 ml glycine-NaOH buffer (pH 10.0, 50 mM), 5 μl crotyl alcohol, and 0.3 g wet cells/ml and were incubated at 30°C for 48 h. After incubation, the concentrations of the substrate and product in each reaction mixture were determined by gas chromatography-mass spectrometry (GC-MS) and GC as previously described (19).

Molecular identification, Biolog phenotype microarray, and biological safety assessment. 16S rRNA gene fragments of the isolated strain WZY002 were amplified from its genomic DNA by PCR using the primer pair 7f (5′-CAGAGTTTGATCCTGGCT-3′) and 1540r (5′-AGGAGGTG ATCCAGCCGCA-3′). The PCR products were purified, and both strands were sequenced. The 16S rRNA gene sequence obtained was compared with sequences in the GenBank database using BLAST, and multiple alignments of the determined 16S rRNA gene sequences and reference sequences obtained from the GenBank database were carried out using the ClustalW software package, version 1.83 (20). A phylogenetic tree was constructed based on the homologous 16S rRNA gene sequences using the software program MEGA5 (21). The Biolog phenotype microarray was tested according to a previously reported procedure (19), with incubation at 33°C for 36 h. The pure culture was sent to the China Center of Industrial Culture Collection (Beijing, China) for testing of antimicrobial susceptibility and hemolysis. Antimicrobial susceptibility testing was carried out using a commercially available Etest protocol (bioMérieux, Marcy l'Etoile, France). In hemolysis testing, the positive and negative controls were *Bacillus subtilis* ATCC 21332 and *Streptococcus agalactiae* ATCC 13813, respectively.

Chemoselective reduction of aldehydes and ketones by the whole cells of *Yokenella* sp. WZY002. The reaction mixture (2 ml) contained 200 mM sodium phosphate buffer (pH 8.0), 50 mM aldehydes or 10 mM ketones, and 0.25 g wet cells, with glucose at a concentration which was 5 times higher than the aldehyde or ketone concentration. Incubation was carried out at 30°C for 12 h unless otherwise specified. After incubation, the reactants in the chemoselective reduction were extracted, dried over anhydrous sodium sulfate, and then analyzed by GC and GC-MS as previously described (19) but with appropriate adjustments of oven temperature programs. The effect of pH on bioreduction was determined over the pH range 6.0 to 9.6 with 200 mM Na_2HPO_4-NaH_2PO_4 solution as a buffer and crotonaldehyde as the substrate. The effect of temperature in the range of 4 to 60°C was determined. The effects of each cosubstrate on reduction were determined with the cosubstrate at a concentration which was 5 times higher than that of the substrate in the reaction mixture. The effect of the glucose concentration on reduction was determined in the range of 0 to 750 mM.

Purification of YsADH. *Yokenella* sp. WZY002 cells (30 g) were suspended in 100 ml Tris-HCl buffer (pH 8.0) and disrupted by sonication (Sonics 500-W/20-kHz ultrasonic processors; Sonics & Materials, Inc., Newtown, CT, USA) while in an ice bath. The disrupted cells were centrifuged at 12,000 × *g* for 10 min. The supernatant was loaded onto a DEAE-Sepharose column (2 by 10 cm) that had been equilibrated with a buffer A (50 mM Tris-HCl, pH 8.0). ADH was eluted with 20% buffer B (50 mM Tris-HCl containing 1 M NaCl, pH 8.0) applied at a rate of 1 ml min^{-1}. Fractions containing ADH activity were pooled and loaded at a flow rate of 1 ml min^{-1} onto a phenyl-Sepharose column (2 by 8 cm) that had been equilibrated with 0.8 M ammonia sulfate in buffer A. A linear gradient (0.82 to 0 M ammonia sulfate in buffer A) was applied at a flow rate of 1 ml min^{-1}. ADH was eluted with ultrapure water. Fractions containing ADH activity were concentrated by ultrafiltration using YM-10 membranes (Millipore Corporation, Bedford, MA, USA). The concentrated samples were applied to a Superdex-200 gel filtration column (2.6 by 60 cm) that had been equilibrated with buffer A at a rate of 1.5 ml min^{-1}. The purity of the fractions containing ADH activity was verified using SDS-PAGE as previously described (22). Active staining of $NADP^+$-dependent ADHs was performed on a 9% native polyacrylamide gel as described elsewhere (23) with some modifications. After electrophoresis, the gels were soaked in a solution containing 0.1 mM phenazine methosulfate, 0.1 mM nitrotetrazolium blue chloride, 10 mM $NADP^+$, 100 mM crotonaldehyde or benzaldehyde, and 50 mM Tris-HCl buffer (pH 8.0), and gently shaken for 10 min at 30°C.

Expression of gene for YsADH in *E. coli* and purification of recombinant YsADH. Purified native YsADH was subjected to 12% SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. A piece of membrane was sent to Sangon Biotech Co., Ltd. (Shanghai, China) for determination of the N-terminal amino acid sequence of the purified enzyme. The gene encoding YsADH, i.e., *adh*, was amplified from the genomic DNA of *Yokenella* sp. WZY002 using the primer pair adhF (5′-ATGTCTATTATAAAAAGCTATGCC-3′) and adhR (5′-TCAAAAG TCGGCTTGCAG-3′). The targeted DNA fragment was ligated with the expression vector through the AT ligation strategy, according to the instruction of the pEASY-E1 expression kit (TransGen Biotech Co., Ltd., Beijing, China). The resulting plasmid, harboring the *adh* gene, was designated pEASY-E1-*adh*.

The plasmid pEASY-E1-*adh* was transformed into *E. coli* BL21(DE3). The recombinant cells were grown at 37°C in LB medium containing 100

μg ml^{-1} of ampicillin until the optical density at 600 nm (OD_{600}) reached 0.6 to 0.8, when gene expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were subsequently cultured at 20°C for 20 h and then harvested by centrifugation. The pellet was washed and resuspended in 50 mM Tris-HCl buffer (pH 8.0) and disrupted by ultrasonication for 8 min. The cell lysate was centrifuged at 12,000 × *g* for 10 min, and the supernatant was applied to a nickel-nitrilotriacetic acid (Ni-NTA) chelating affinity column (Bio-Rad Laboratories, Hercules, CA, USA) that had been equilibrated with binding buffer (5 mM imidazole and 300 mM NaCl dissolved in 50 mM Tris-HCl, pH 8.0). The bound enzyme was eluted by applying a stepwise gradient of imidazole at concentrations from 10 to 250 mM. The fractions containing YsADH eluted at the concentration of 100 mM imidazole were desalted with 50 mM Tris-HCl (pH 8.0) by ultrafiltration using YM-10 membranes and then stored at −20°C for further use.

ADH assay. Activities of native and recombinant YsADHs were measured at 55°C or 65°C by monitoring the change in A_{340}. Unless otherwise specified, the enzyme assay for alcohol oxidation was carried out in duplicate in a reaction mixture (2.5 ml) composed of 20 mM crotyl alcohol and 1 mM $NADP^+$ in 50 mM Tris-HCl (pH 8.0) buffer. The assay mixture (2.5 ml) for the reduction of ketone/aldehyde contained 10 mM crotonaldehyde and 0.3 mM NADPH in 50 mM 2-(*N*-morpholino)ethanesulfonic acid buffer (MES) (pH 6.5). The reaction was started by the addition of the enzyme (3 μg). One unit of the activity is defined as formation or oxidation of 1 μmol NADPH per min. The protein concentrations of all samples were determined using the Bradford method (24), with bovine serum albumin as the standard.

Characterization of recombinant YsADH. The molecular weight of recombinant YsADH in the native form was determined using a high-performance liquid chromatography apparatus equipped with a size exclusion column, Wat011535 (Waters Corporation, Milford, MA, USA), in which the flow phase was 50 mM phosphate buffer containing 150 mM NaCl (pH 6.8). The molecular weight standards consist of thyroglobulin (670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B_{12} (1,350).

The effect of temperature on the enzyme activity was determined at temperatures from 20 to 75°C. To investigate the thermostability of YsADH, the residual activity was measured by the standard assay for crotyl alcohol oxidation with incubation at 55°C, 60°C, and 65°C for appropriate time intervals. The optimal pHs for aldehyde/ketone reduction and alcohol oxidation were determined using 50 mM MES (pH 5.5 to 8.0) and Tris-HCl (pH 7.0 to 9.0) buffers, respectively. Substrate specificity was determined using allylic and aromatic alcohols, aldehydes, or ketones under standard assay conditions. The effects of cations, EDTA, dithiothreitol (DTT), and iodoacetate on enzyme activity were examined by adding each compound at a final concentration of 1 mM. The residual enzyme activity was determined by measuring the oxidation of crotyl alcohol in 50 mM Tris-HCl (pH 8.0), with determination of enzyme activity in the absence of a test compound as the control.

Enzyme kinetic parameters were determined using different substrates and $NADP^+$ or NADPH. Various substrate concentrations were used for determining the activities at 65°C for aldehyde reduction and 55°C for alcohol oxidation when concentrations of the appropriate coenzymes were constant. Substrates and coenzymes used were $NADP^+$ (0 to 1.44 mM), crotyl alcohol (0 to 112.5 mM), benzyl alcohol (0 to 192.4 mM), NADPH (0 to 0.45 mM), crotonaldehyde (0 to 72.8 mM), and benzyl aldehyde (0 to 15.7 mM). Apparent values of K_m, the maximum initial velocity (V_{max}), and the substrate inhibition constant, K_{is}, were calculated by fitting the data into the equation $v = V_{max}[S]/([S] + K_m + [S]^2/K_{is})$, where $[S]$ is the substrate concentration, using the software program Polymath 6.0 (25, 26).

Nucleotide sequence accession numbers. The gene encoding YsADH and the 16S rRNA gene sequence of *Yokenella* sp. WZY002 have been deposited in the GenBank database under the accession numbers KF887947 and KF874625, respectively.

RESULTS

Identification of the newly isolated strain WZY002. To isolate the desired strains capable of oxidizing allylic alcohols, the NAD(P)H fluorescence-based screening method (19) was employed as a high-throughput screening strategy. Fifty isolates obtained from soil samples through an enrichment cultivation procedure were examined, and 24 isolates harboring crotyl alcohol oxidizing activity were selected. One strain, marked as WZY002, showed the highest allylic alcohol-oxidizing activity and was finally chosen for further investigation. Colonies of WZY002 on nutrient agar appeared light yellow, round, smooth, mucoid, and translucent. The aerobic cells were rod shaped, Gram negative, and nonsporulating. The 16S rRNA gene sequence of 1,431 bp had significant homology with sequences from *Yokenella regensburgei* strain CIP 105435 (1,417/1,431 identity; JN175339) and *Y. regensburgei* strain NBRC 102600 (1,416/1,431 identity; AB681877) (Fig. 1). Carbon source utilization indicated that strain WZY002 had a high probability of being *Y. regensburgei* despite relatively low similarity (0.306). Therefore, the strain used for the study was designated *Yokenella* sp. WZY002. Antimicrobial susceptibility and hemolysis testing was performed to assess the biological safety of *Yokenella* sp. WZY002. The results of antimicrobial susceptibility testing indicated that *Yokenella* sp. WZY002 was sensitive to antibiotics, such as tetracycline, nitrofurantoin, gentamicin, ceftazidime, chloromycetin, norfloxacin, and kanamycin, whereas it showed resistance to benzylpenicillin, with a minimum inhibition concentration of 32 μg ml^{-1} (see Table S1 in the supplemental material). In addition, the organism was nonhemolytic.

Chemoselective reduction of α,β-unsaturated aldehydes/ketones by the whole cells of *Yokenella* sp. WZY002. In reaction mixtures containing 50 mM α,β-unsaturated aldehydes incubated at 30°C and pH 8.0, the addition of 250 mM glucose completely inhibited the formation of α,β-unsaturated carboxylic acids and significantly improved the yield of the desired α,β-unsaturated alcohols (see Fig. S1 in the supplemental material). Reduction of 50 mM crotonaldehyde gave crotyl alcohol with a yield of 96.1%. The strain showed similarly high activities with the other allylic aldehydes except when tested with cinnamaldehyde (Table 1). The reductions of allylic ketones were catalyzed at much lower rates, with the formation of 3-octen-2-ol from 3-octen-2-one being undetectable. Most of the reduction reactions yielded α,β-unsaturated alcohol as the sole product, except that the reduction of (E)-oct-2-enal (entry 4 in Table 1) led to the formation of *trans*-2-octen-1-ol (68.7% yield) and its derivative acetic acid oct-2-enyl ester (27.0% yield). In addition, benzaldehyde and vanillin (3-methoxy-4-hydroxybenzaldehyde) were reduced to benzyl alcohol and vanillic alcohol, respectively (entries 1 and 2 in Table 2). The yields for reductions of aromatic ketones were not satisfactory. Reductions of aromatic ketones catalyzed by *Yokenella* sp. WZY002 were stereoselective for formation of (*S*)-enantioselective alcohols. Thus, the reduction of 2-hydroxyacetophenone (entry 6 in Table 2) resulted in formation of (*S*)-1,2-phenylethanediol with an enantiomeric excess (e.e.) value of >99.9%.

Cloning and overexpression of the gene encoding YsADH. The subunit size of the alcohol dehydrogenase as determined by SDS-PAGE was 37 ± 1 kDa (Fig. 2A). The specific activity of the purified enzyme in crotyl alcohol oxidation was 79.6 U mg^{-1}, and recovery was 18.3% (Table 3). The active staining for allylic/benzyl alcohol

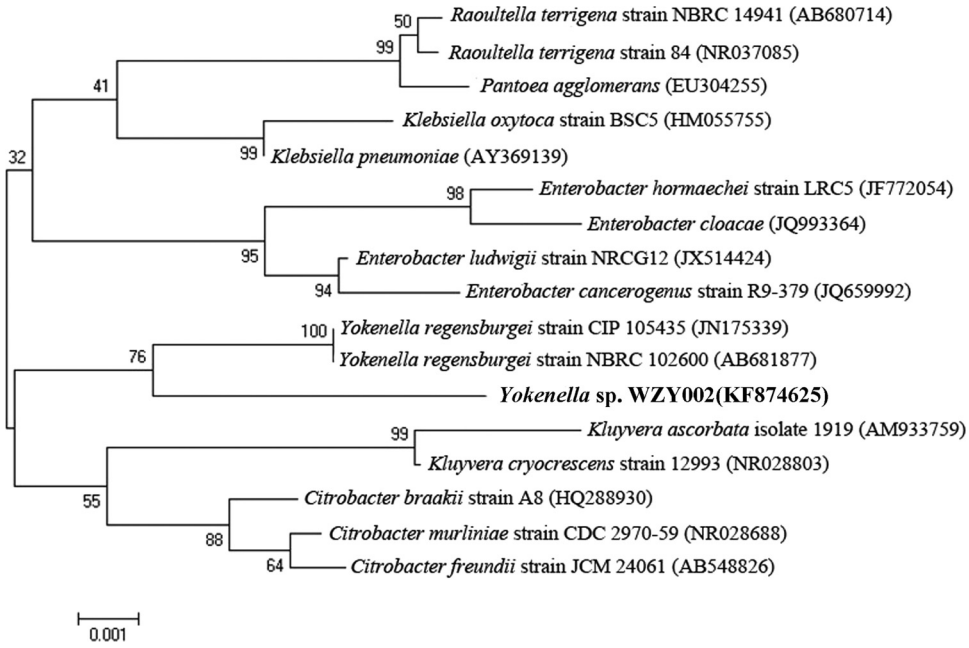


FIG 1 Phylogenetic analysis of *Yokenella* sp. WZY002 with related strains based on the 16S rRNA gene sequences. The sequences were aligned using the ClustalW software program, and subsequently a phylogenetic tree was constructed against 1,431-bp fragments shared by all the sequences using the neighbor-joining method. The number of bootstrap replications was set at 1,000. The scale bar represents 0.001 substitutions per sequence position. Numbers in parentheses are accession numbers of published sequences.

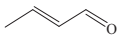
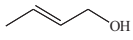
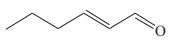
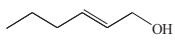
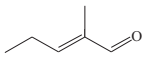
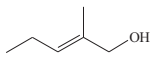
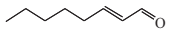
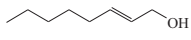
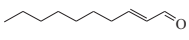
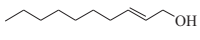
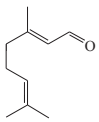
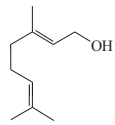
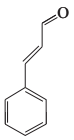
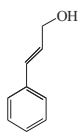
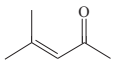
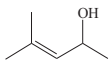
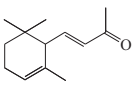
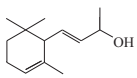
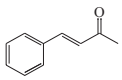
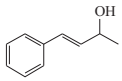
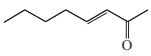
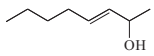
dehydrogenase activities showed that YsADH activity was predominant and that multiple isoenzymes of YsADH were present in the crude extract of *Yokenella* sp. WZY002 (Fig. 2B). The amino-terminal (N-terminal) sequence of YsADH was determined to be SIIKSYAAK EAGSEL. This was 100% identical to the N-terminal sequence of a hypothetical protein from *Citrobacter koseri* ATCC BAA-895 (GenBank accession no. ABV14613). According to the gene sequence of the homologous enzyme in *Citrobacter koseri*, a set of primers was designed and the gene encoding YsADH was fully sequenced; its product consisted of 339 amino acid residues with a calculated molecular mass of 36,411 Da. The deduced amino acid sequence of YsADH showed high overall identity to putative zinc-containing ADHs from enterobacteria, e.g., ADHs from *Citrobacter koseri* ATCC BAA-895 (96% identity; ABV14613), *Shigella flexneri* 1235-66 (95% identity; EIQ53054), *Enterobacter cancerogenus* ATCC 35316 (93% identity; EFC54248), *Salmonella bongori* N268-08 (92% identity; AGR61658), and *Escherichia coli* MS16-3 (91% identity; ADD59510). The structure-related alignment revealed that YsADH had all conserved residues for the binding of catalytic and structural zincs (25). The structural zinc binding site was made up of four closely spaced cysteines (Cys96, Cys99, Cys102, and Cys110), while the residues Cys41, His63, and Cys152 were critical for the coordination of the catalytic zinc (Fig. 3). Moreover, the motifs of putative active site and $NADP^+$ binding were also identified to be $G_{62}H_{63}E_{64}X_2G_{67}X_5G_{73}$ and $G_{176}XG_{178}XXG_{181}$, respectively (27).

The enzyme was synthesized in cells of a recombinant *E. coli* BL21(DE3) strain harboring pEASY-E1-*adh*. After the induction with 0.6 mM IPTG at 28°C, neither soluble YsADH nor YsADH activity was detected in the crude extracts of *Yokenella* sp. WZY002; meanwhile, the insoluble aggregates of expressed proteins were observed. When both the induction temperature and IPTG concentration were decreased, to 20°C and 0.1 mM, respectively, the specific activity of YsADH in the crude extracts was increased up to 2.63 U mg^{-1} in the crotyl alcohol oxidation, and the amount of YsADH in the soluble form constituted of around 5% of total heterogeneously expressed proteins. The soluble recombinant YsADH as an N-terminal histidine-tagged fusion protein was purified, and its specific activity was comparable to that of native YsADH in either crotyl alcohol oxidation or crotonaldehyde reduction. The SDS-PAGE analyses of purified recombinant YsADH yielded a single band with a molecular mass of 39 ± 1 kDa (Fig. 2A), and its native molecular mass was determined to be 75 ± 3 kDa using gel filtration, suggesting that recombinant YsADH in native form appeared to be a homodimer.

Catalytic properties. The enzyme was NADP(H) dependent. The activity using NAD(H) as a coenzyme was undetectable. The enzyme was thermoactive, and its optimal values of temperature for crotyl alcohol oxidation and crotonaldehyde reduction were 55 and 65°C, respectively (Fig. 4A). The enzyme demonstrated the highest activity at pH 8.0 for crotyl alcohol oxidation, while the optimal pH for crotonaldehyde reduction was found to be pH 6.5 (Fig. 4B). Under the optimized conditions, the highest activity of crotonaldehyde reduction was 5.4 times higher than that of crotyl alcohol oxidation.

Activity of allylic/benzyl alcohol dehydrogenases is susceptible to inhibition by metal ions or thiol-blocking reagents (14, 28). Thus, the effects of metal ions and chemical reagents on the activity of recombinant YsADH were investigated (Table 4). The addition of EDTA, DTT, or Ba^{2+} to the assay mixture increased the activity of YsADH by 110.4 to 123.1%, while most of the tested cations, such as Ca^{2+}, Na^+, Mg^{2+}, Mn^{2+}, and K^+, had no significant effect on activity. On the other hand, activity was slightly inhibited by Al^{3+} (13.5% inhibition) and strongly inhibited by Fe^{2+} (39.7%), Co^{2+} (80.5%), Zn^{2+} (99.5%), Ag^+

TABLE 1 Substrate spectrum of *Yokenella* sp. WZY002 and recombinant YsADH against allylic aldehydes and ketones

Entry	Substrate	Product	Whole-cell catalysis		Relative activity of recombinant YsADH (%)[a]
			Time (h)	Yield (%)[a]	
1			12	96.1 ± 0.07	100[b] ± 4.5
2			12	98.4 ± 0.2	41.7 ± 9.4
3			12	94.9 ± 0.5	41.5 ± 8.2
4			12	68.7 ± 5.7	30.2 ± 5.8
5			12	90.9 ± 2.1	16.7 ± 2.9
6			12	87.4 ± 0.6	37.4 ± 1.0
7			12	9.5 ± 3.7	ND[c]
8			17	3.6 ± 0.3	3.0 ± 1.5
9			17	3.0 ± 0.1	ND[c]
10			17	4.8 ± 0.7	ND[c]
11			17	ND[c]	7.9 ± 0.6

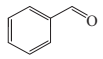
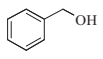
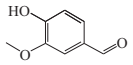
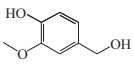
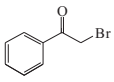
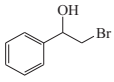
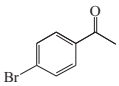
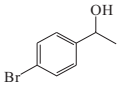
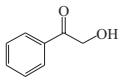
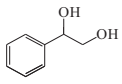
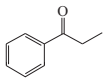
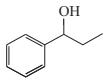
[a] Means ± SD.
[b] The relative activity of 100% in aldehyde/ketone reduction means 399.2 U mg^{-1}.
[c] ND, not detectable.

(100%), and Cu^{2+} (100%). In addition, iodoacetate at concentrations of 1 mM and 10 mM had a small inhibitory effect on the activity.

Substrate spectrum. The substrate specificity of recombinant YsADH was determined using the same set of alcohols, aldehydes, and ketones as for the whole cells of *Yokenella* sp. WZY002 as biocatalysts (Tables 1 and 2). In the reduction reaction, the enzyme exhibited the highest activity (427 U mg^{-1}) for benzyl aldehyde as the substrate, next to that for crotonaldehyde (399 U mg^{-1}). In contrast to the substrate specificity of the whole cells of *Yokenella* sp. WZY002, the enzyme could not reduce cinnamaldehyde and ketones, including α-ionone, *trans*-4-phenyl-3-buten-2-one, vanillin, acetophenone, and 2-hydroxyacetophenone. In the alcohol oxidation, the enzyme showed the highest activity (79.9 U mg^{-1}) on crotyl alcohol, and the benzyl alcohol oxidizing activity was 41.7% of that for crotyl alcohol. The enzyme was also active toward saturated alcohol, such as 1-propanol and 1-butanol. The activities for 1-propanol and 1-butanol were 17.4% and 56.0% of that for crotyl alcohol, respectively.

Tolerance to heat and organic solvents. The enzyme was not only thermoactive but also thermostable. The thermostability of the purified enzyme was investigated by determining its residual activities when the enzyme samples were incubated at 55, 60, and 65°C (Fig. 5). The half-life values at 55 and 60°C were

TABLE 2 Substrate spectrum of *Yokenella* sp. WZY002 and recombinant YsADH against aromatic aldehydes and ketones

Entry	Substrate	Product	Whole-cell catalysis			Relative activity of recombinant YsADH[a,b] (%)
			Time (h)	Yield (%)[a]	Selectivity (%)[a,c]	
1			12	99.6 ± 0.05		107 ± 2.9
2			12	88.4 ± 1.3		ND[d]
3			24	10.0 ± 0.7	91.1 ± 0.7	ND[d]
4			24	4.8 ± 0.5	61.7 ± 2.2	27.4 ± 1.7
5			24	12.1 ± 1.0	95.5 ± 1.4	1.1 ± 0.4
6			24	4.9 ± 0.4	>99.9	ND[d]
7			24	ND[d]		ND[d]

[a] Means ± SD.
[b] The relative activity of 100% in aldehyde/ketone reduction means 399.2 U mg^{-1}.
[c] The enantiomeric excess of the product was (*S*)-enantioselective.
[d] ND, not detectable.

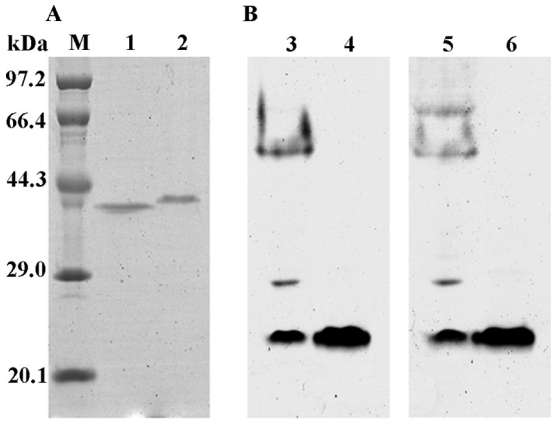


FIG 2 SDS-PAGE analysis of the purified YsADHs (A) or active staining of alcohol dehydrogenase activities after native PAGE (B). M, molecular markers; lane 1, native YsADH from *Yokenella* sp. WZY002; lane 2, recombinant YsADH. Crude cell extracts of *Yokenella* sp. WZY002 (lanes 3 and 5) and purified native YsADH (lanes 4 and 6) were used for native PAGE. Crotyl alcohol and benzyl alcohol were used as the substrates for active staining in lanes 3 and 4 and lanes 5 and 6, respectively.

determined to be 6.2 and 1.5 h, respectively, revealing its moderately thermostable feature. The activity loss at 65°C was relatively rapid, and the enzyme retained only 30.8% of the initial activity after 30 min of heat treatment. The effect of organic solvents on activity was examined by adding each organic solvent to the reaction mixture. The presence of 20% (vol/vol) acetone improved the enzyme activity by 125%, while a relatively high activity (91% of the activity without a solvent) was observed in the case of 20% (vol/vol) ethanol (Fig. 6A). The enzyme retained >49% of the initial activity in 20% (vol/vol) solvents, including methanol, ethanol, dimethyl sulfoxide

TABLE 3 Purification of allylic/benzyl alcohol dehydrogenase from *Yokenella* sp. WZY002

Purification step	Total proteins (mg)	Total activity (U)	Sp act[a] (U mg^{-1})	Purification (fold)	Yield (%)
Crude cell extract	8.1×10^2	226	0.279	1.00	100
DEAE-sepharose	22	81.4	3.70	13.0	36.0
Phenyl-sepharose	1.0	57.2	57.2	204	25.3
Gel filtration	0.50	39.8	79.6	284	17.6

[a] Activity was examined for the oxidation of crotyl alcohol in the Tris-HCl buffer (50 mM, pH 8.0).

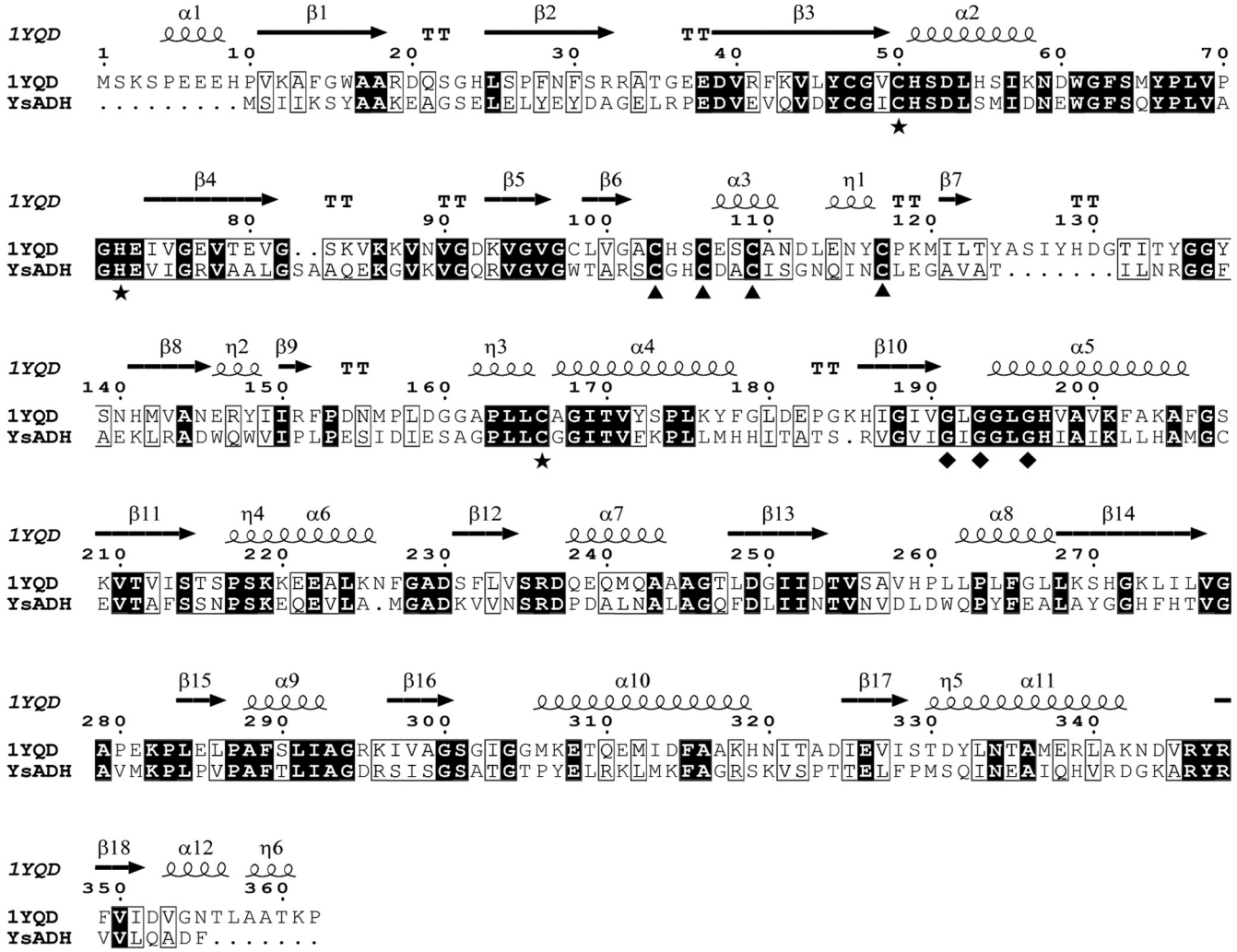


FIG 3 Structure-related sequence alignment between YsADH and its close homologue: sinapyl alcohol dehydrogenase from *Populus tremuloides*. The homologue of YsADH, sinapyl alcohol dehydrogenase from *Populus tremuloides*, with a PDB code of 1YQD, was identified by performing a BLASTP search (38), and the alignment was carried out using the program ClustalW. The sequence alignment was subsequently visualized using ESPript 2.2 (39). Above the alignments are elements of the secondary structure of 1YQD. The numbering shown is from 1YQD. Diamonds, critical coenzyme binding residues; stars, putative residues involved in the coordination of catalytic zinc; triangles, putative residues responsible for the coordination of structural zinc. Strictly conserved residues are highlighted with black boxes.

(DMSO), and isopropanol and remained active in 40% (vol/vol) of all examined solvents. In comparison with most of the water-miscible solvents, the enzyme exhibited higher activity in 50% (vol/vol) of water-immiscible solvents. To further investigate its stability in the presence of miscible organic solvents, the enzyme was incubated with 20% (vol/vol) organic solvent at 30°C. After 24 h of incubation, the residual activities in DMSO, methanol, and acetone kept 74.3 to 87.5% of the initial activity; meanwhile, the solvent tolerance in ethanol and isopropanol was relatively poor (7.7 to 23.4% of the initial activity) (Fig. 6B).

Kinetic parameters. YsADH nearly obeyed Michaelis-Menten kinetics at low substrate concentrations, but the activity of YsADH declined with increased substrate concentrations. The substrate inhibition constant values for crotyl alcohol and benzyl alcohol were about 5.0 and 3.3 times greater than those for crotonaldehyde and benzaldehyde (Table 5), suggesting that YsADH had higher tolerance to alcohols than to aldehydes. In the interconversion between aldehyde and its corresponding alcohol, the enzyme had higher catalytic efficiency and lower K_m for crotonaldehyde and benzaldehyde than for crotyl alcohol and benzyl alcohol. The apparent K_m values for NADPH and $NADP^+$ were comparable, whereas the specificity constant K_{cat}/K_m for NADPH as an electron donor in the aldehyde reduction (720 s^{-1} mM^{-1}) was 2.6 times higher than that for the electron acceptor $NADP^+$ in the oxidation of the corresponding alcohol (277 s^{-1} mM^{-1}). These catalytic properties suggest that the enzyme was more likely to be involved in aldehyde reduction coupled with oxidation of NADPH rather than alcohol oxidation coupled with reduction of $NADP^+$ *in vivo*.

DISCUSSION

The strain used in this study can reduce a wide range of aldehydes and ketones to allylic alcohols, such as nerol, geraniol, cinnamyl

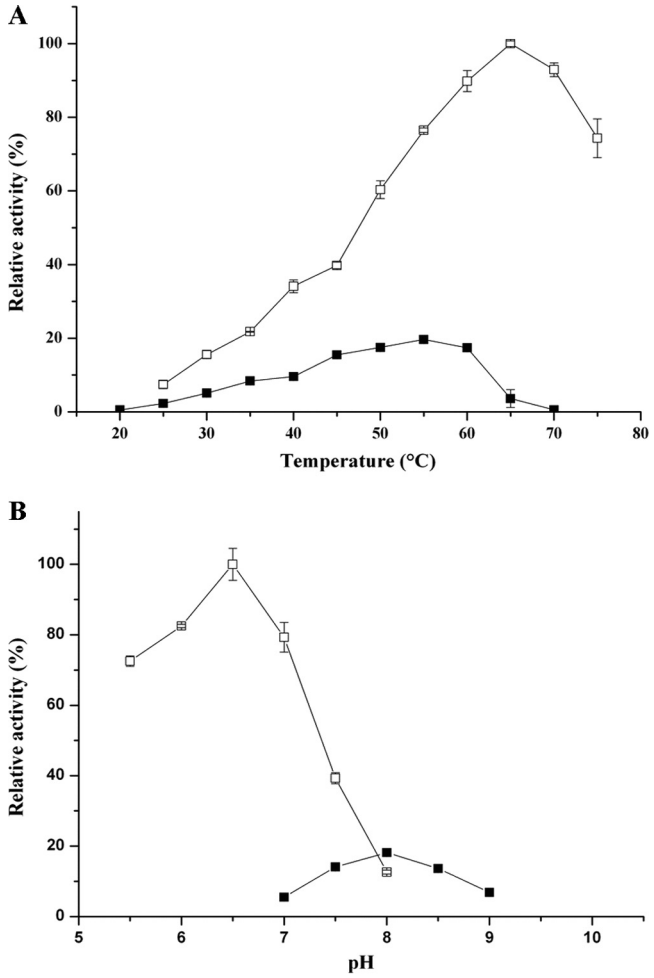


FIG 4 Effect of temperature (A) or pH (B) on the activities of recombinant YsADH. Relative activity of 100% represents 399.2 U mg^{-1} for crotonaldehyde reduction at 65°C and pH 6.5. Solid symbols, oxidation; empty symbols, reduction.

alcohol, and vanillyl alcohol, which are widely used for flavoring in food industry (17). In addition to its activity with allylic aldehydes, the organism can reduce acetophenone and its derivatives to chiral aromatic alcohols, some of which are valuable intermediates for synthesis of biologically or pharmacologically active compounds (29). Bioreduction catalyzed by whole cells of *Yokenella* sp. WZY002 required no external coenzyme regeneration, and the internal regeneration of coenzyme for bioreduction was substantially improved by the addition of glucose. To the best of our knowledge, this is the first report of the use of a *Yokenella* species for synthesis of allylic and benzyl alcohols. The substrate spectrum of *Yokenella* sp. WZY002 was broader than that of recombinant YsADH. This difference indicated the presence of other alcohol dehydrogenases in *Yokenella* sp. WZY002, including a chiral alcohol dehydrogenase for asymmetrical reduction of 2-hydroxyacetophenone. Furthermore, the active staining of an electrophoresis gel for alcohol dehydrogenase activity in cell extracts showed different patterns against crotonaldehyde and benzyl aldehyde as the substrate, indicating that it is very likely to have multiple isoenzymes with allylic/benzyl alcohol dehydrogenase activities. Therefore, *Yokenella* sp. WZY002 could serve as a good resource for exploring alcohol dehydrogenases as potential biocatalysts.

TABLE 4 Effects of chemical compounds on activity of recombinant YsADH

Chemical (concn, mM)	Relative activity (%)[a]
None	100 ± 6.2
DTT (1)	123 ± 2.3
$BaCl_2$ (1)	113 ± 0.8
EDTA (1)	110 ± 4.2
$CaCl_2$ (1)	105 ± 6.0
NaCl (1)	105 ± 4.2
$MgCl_2$ (1)	98.9 ± 4.0
$MnCl_2$ (1)	96.8 ± 0.3
KCl (1)	94.0 ± 5.9
$AlCl_3$ (1)	86.5 ± 0.8
$FeSO_4$ (1)	60.3 ± 17
$CoCl_2$ (1)	19.5 ± 0.6
$ZnCl_2$ (1)	0.5 ± 0.3
$AgNO_3$ (1)	ND[b]
$CuSO_4$ (1)	ND[b]
Iodoacetate (1)	95.9 ± 2.1
Iodoacetate (10)	94.5 ± 1.8

[a] Activity was examined for the oxidation of crotyl alcohol in Tris-HCl buffer (50 mM, pH 8.0). Relative activity of 100% equals 79.9 U mg^{-1}. Values are means ± SD.
[b] ND, not detectable.

Side reactions are often encountered in whole-cell biocatalysis due to the presence of competing enzymes. When no glucose was added to the reaction mixture, aldehyde dehydrogenase acted as the major competing enzyme, which led to the undesired oxidation of the carbonyl group in aldehydes. The oxidation of allylic aldehydes was completely avoided by the addition of a higher concentration of glucose to the reaction mixture. In the presence of 250 mM glucose, the aldehyde/ketone reduction generally demonstrated excellent chemoselectivity. Moreover, none of the corresponding saturated compounds (aldehydes, ketones, or alcohols) was identified, suggesting that whole cells of *Yokenella* sp. WZY002 did not exert interfering enoate reductase activity under the conditions employed. In the case of (E)-oct-2-enal reduction,

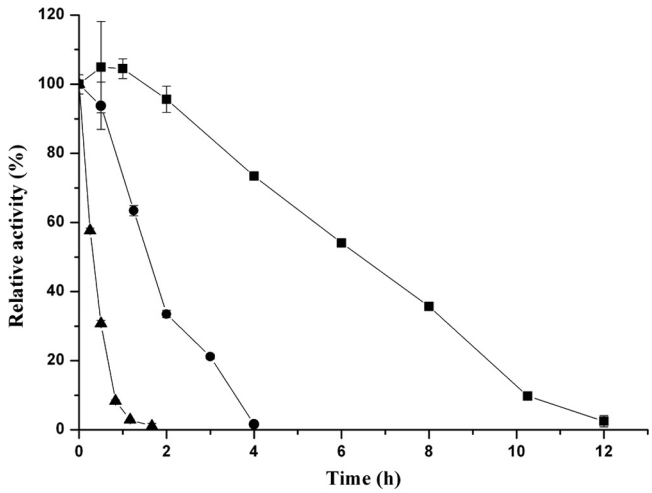


FIG 5 Thermostability of recombinant YsADH. The relative activity of 100% represents 79.9 U mg^{-1} for the oxidation of crotyl alcohol at 55°C and pH 8.0. Squares, 55°C; circles, 60°C; triangles, 65°C.

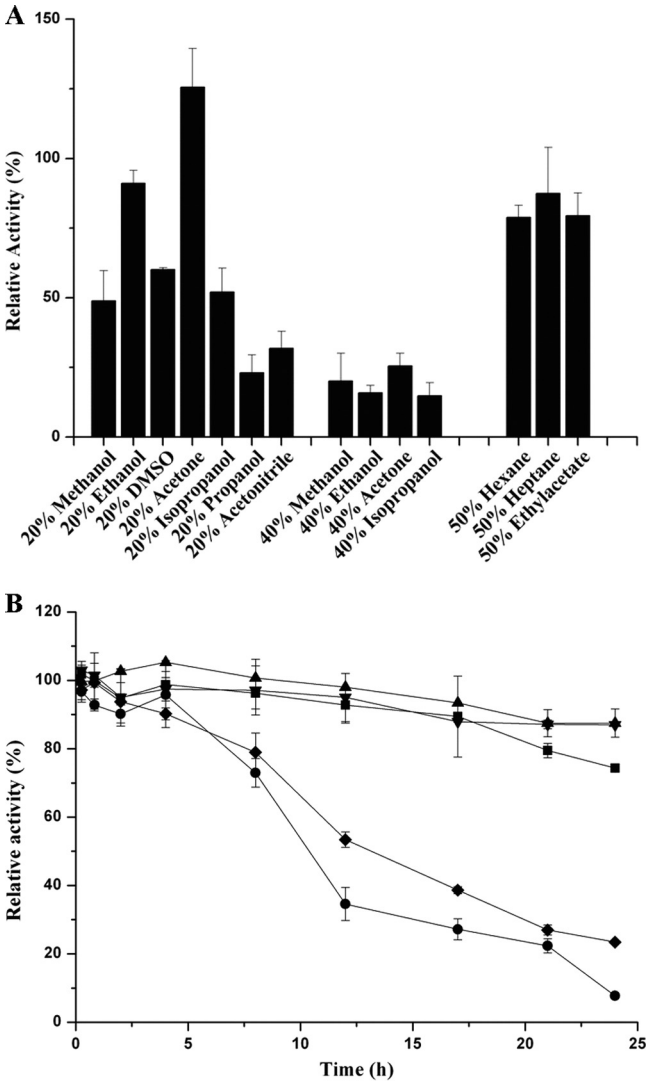


FIG 6 Effect of organic solvents on the activity (A) or stability (B) of YsADH. (A) Activity was examined for crotyl alcohol oxidation with organic solvents in the Tris-HCl buffer (50 mM, pH 8.0) at 30°C. Relative activity of 100% corresponds to 21.3 U/mg when no solvent was added to the reaction mixture. (B) Stability was determined by measuring the residual activity at 55°C and pH 8.0 after incubation with 20% (vol/vol) organic solvents at 30°C. Squares, methanol; circles, ethanol; vertical triangles, acetone; inverted triangles, DMSO; diamonds, isopropanol. Relative activity of 100% equals 79.9 U mg^{-1} in crotyl alcohol oxidation.

the yield of the desired *trans*-2-octen-1-ol was partially concealed by the presence of esterase activity, which led to the formation of its ester derivatives from *trans*-2-octen-1-ol. In contrast to whole cells, isolated enzymes as a biocatalyst should provide significant benefit, since no side reactions should occur (7). Thus, purification and characterization of YsADH would be helpful to overcome the limitations of the whole-cell biocatalysis.

BLAST searches of the YsADH amino acid sequence indicated that its homologues with >90% identity are prevalent in the family *Enterobacteriales*; none of these have been characterized with respect to biocatalysis. Among the homologues deposited in the PDB database, the amino acid sequence of YsADH showed the highest identity (37%) to that of sinapyl alcohol dehydrogenase from the plant *Populus tremuloides* (PDB no. 1YQD). Despite low sequence similarity, the structure-related alignment showed that the amino acid sequence of YsADH exhibits all the characteristics of medium-chain zinc-dependent alcohol dehydrogenases, including the binding motifs of NADP(H) and catalytic and structural zincs (14, 25). YsADH also had features distinct from those of other allylic/benzyl alcohol dehydrogenases (13–16). The amino acid sequence of native YsADH started with serine, indicating that N-terminal methionine was excised in the mature enzyme. The role of N-terminal methionine excision remained elusive, since the activity of recombinant YsADH with initial methionine was nearly identical to that of the native one. Similar to the aryl alcohol dehydrogenase from *Acinetobacter baylyi* ADP1, YsADH was highly sensitive to inactivation by Ag^{+}, Cu^{2+}, and Zn^{2+}, suggesting the importance of sulfhydryl groups for the activity of the enzyme (14). Consistently, DTT, which prevents oxidation of the thiol group, maximally enhanced the activity by 123.1%, whereas the activity was slightly susceptible to iodoacetate as a thiol-blocking agent. Among the all eight cysteine residues of YsADH, Cys41 and Cys152 are involved in the binding of catalytic zinc, and the four cysteine residues at position 96, 99, 102, and 110 may bind structural zinc. Thus, the thiol groups of the other two cysteines, Cys38 and Cys193, might play an important role in biocatalysis. In addition, overexpressed YsADH was prone to aggregate, and the formation of inclusion bodies could be partly avoided by lowering the induction temperature and IPTG concentration. Similar to the case with coniferyl alcohol dehydrogenase from *Streptomyces* sp. NL15-2K (30), heat shock treatment of recombinant *E. coli* improved the solubility of recombinant YsADH at the expense of lower specific activity (Y. Wang and X. Ying, unpublished results), which warrants further investigation.

As a member of the NAD(P)-dependent alcohol dehydrogenase family, YsADH was expected to preferentially catalyze the oxidation of alcohols into aldehydes. However, the enzyme cata-

TABLE 5 Kinetic parameters of recombinant YsADH[a]

Substrate (concn, mM)	Cosubstrate (concn, mM)	Apparent V_{max} (U mg^{-1})	Apparent K_m (mM)	Apparent K_{is}[b] (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})
Crotyl alcohol	$NADP^+$ (0.4)	156.7 ± 3.8	9.1 ± 0.8	229 ± 3.2	101 ± 2.5	11.0 ± 1.0
Benzyl alcohol	$NADP^+$ (0.4)	145.1 ± 9.0	14 ± 1.6	101 ± 1.5	93.3 ± 5.8	6.6 ± 0.9
$NADP^+$	Crotyl alcohol (40)	301.2 ± 21	0.7 ± 0.1		194 ± 14	278 ± 44
Crotonaldehyde	NADPH (0.4)	629.4 ± 25	3.3 ± 0.2	45.4 ± 5.7	405 ± 16	122 ± 8.9
Benzaldehyde	NADPH (0.4)	559.4 ± 4.1	0.4 ± 0.1	30.5 ± 2.3	360 ± 2.6	899 ± 6.5
NADPH	Crotonaldehyde (4.9)	672.7 ± 17	0.6 ± 0.1		433 ± 0.5	720 ± 19

[a] Means ± SD.
[b] K_{is}, substrate inhibition constant.

lyzed the reduction of aldehydes into alcohols at a higher rate. The values of K_{cat}/K_m for benzyl aldehyde and crotonaldehyde were 136- and 11-fold higher than those for benzyl alcohol and crotyl alcohol, respectively. Therefore, YsADH might be better designated an aldehyde reductase than an alcohol dehydrogenase. Among the tested substrates in the reduction reaction, YsADH preferentially catalyzed aliphatic allylic aldehydes and benzyl aldehyde as the substrate, whereas the enzyme activity with both allylic and aromatic ketones was relative low. YsADH had different activities for different substrates, with that for crotonaldehyde (2-butenal) being greater than that for 2-hexenal, which is greater than that for 2-octenal, which is greater than that for 2-decenal, indicating that the steric hindrance associated with bulky β-substituent of the C═C bond had a considerable effect on the activity (1). The importance of the allylic double bond for the activity was suggested by comparing the oxidizing activities of YsADH for crotyl alcohol and its nonallylic analog 1-butanol. The activity of 1-butanol oxidation was only 56% of that of crotyl alcohol oxidation.

Most thermostable alcohol dehydrogenases that have been characterized are from thermophilic bacteria and archaea, such as the genera of *Thermotoga* and *Thermococcus* (27, 31–33). It should be noted that mesophilic microorganisms, from which few thermoactive alcohol dehydrogenases have been identified, are also an important resource (34, 35). The enzyme was active within a broad temperature range, from 25 to 75°C, in the reduction reaction, while its apparent V_{max} value for crotonaldehyde reduction was as high as 629.4 ± 25.2 U mg^{-1} at 65°C, which was remarkably higher than those of other allylic/benzyl alcohol dehydrogenases characterized (13–16). The enzyme retained 54.5% of the initial activity after 6 h of heat treatment at 55°C, and this moderate thermostability makes it commercially more attractive than its mesophilic counterparts (36).

It was previously reported that the activities of benzyl alcohol dehydrogenases declined as the substrate concentration increased (25, 37). The K_{is} value of YsADH for benzyl aldehyde was 2 to 3 orders of magnitude greater than those of its characterized homologues, indicating higher resistance to substrate inhibition. YsADH displayed high tolerance to not only substrates but also hydrophobic and hydrophilic solvents. YsADH retained its activity for all the miscible solvents examined and exhibited the highest activity in miscible H_2O–20% acetone. Some water-soluble solvents, such as ethanol and 1-propanol, served as the substrate for YsADH, which makes substrate-coupled cofactor regeneration feasible. In addition, YsADH also showed high activity for all the immiscible solvents examined, which can be used as the substrate reservoir and product sink to avoid substrate or product inhibition (36). Given the importance of activity and stability, YsADH could serve as a robust biocatalyst in the synthesis of α,β-unsaturated alcohols from allylic aldehydes.

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